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Normal Human Urinary Porphyrins

By A. COMFORT, H. MOORE and M. WEATHERALL
Department of Pharmacology, London Hospital Medical College, London, E. 1

(*Received* 11 *March* 1954)

Chromatographic methods have recently revealed an extensive intermediary metabolism of the porphyrins, and tri-, penta-, hexa- and hepta-carboxylic porphyrins have been found in pathological urines and other materials (Nicholas & Rimington, 1949, 1951). Less is known about the occurrence of these substances in normal mammalian tissues and excreta and the presence of uroporphyrin in normal material is only now becoming established. Several authors have reported the presence of an ether-insoluble porphyrin fraction in normal urine (Fischer & Zerweck, 1924; Lockwood, 1953) and shown that it probably contains uroporphyrin (Nicholas & Rimington, 1949). Schwartz (1953) has recently referred briefly to the finding of crystallizable uroporphyrin and of a number of intermediate porphyrin fractions in normal human urine. There are also negative reports (Grotepass, 1938; Brachvogel, 1950), but the methods of these authors appear to be less sensitive. In order to study the nature of the non-coproporphyrin fractions in urine, we have examined the porphyrins in the urine of healthy adult male humans and rabbits. Some of our findings have already been reported briefly (Weatherall & Comfort, 1952; Comfort & Weatherall, 1954).

METHODS

Collection of urine. Nine batches of human urine (total 118·4 l.) were obtained (Table 1). Two batches (nos. 65 and 76) were composed of the collected urine of a single individual. The urine was frozen at −20° as soon as it was passed and was stored at this temperature until it was extracted. Three batches (nos. 77, 78 and 79), collected from ten individuals, were kept at room temperature and were extracted within 48 hr. of starting the collection. All the individuals who contributed to these batches were to the best of their knowledge healthy, and (except during the collection of batch 79, where the contributors were not subject to any restriction) they consumed no alcohol and no medicines during and for 24 hr. before the period of collection. The other batches of urine (nos. 69, 74, 80 and 81) were obtained from bottles in a students' lavatory. There was no control over the contributors, and each portion of the batch was extracted within 24 hr. of beginning the collection.

Urine was collected from rabbits kept in galvanized metal metabolism cages and fed on bran, oats and cabbage. Faeces were separated by a grid of suitable mesh in the receiving area of the cages, and the urine was stored at 7° for not longer than 3 days before extraction. Dried extracts were subsequently combined to complete each batch as shown in Table 1.

Extraction of porphyrins from urine. Apart from allowing the urine to stand in daylight at room temperature during the period of collection (or the period of thawing for the

Table 1. *Sources of urine*

Species	Batch no.	Volume (litres)	No. of contributors	Duration of collection (days)	Remarks
Human	65	7·2	1	14	Frozen at −20° as soon as passed. Contributor consumed no alcohol nor medicines
	69	10·0	Unknown	2	No control over contributors
	74	19·3	Unknown	3	As batch 69
	76	14·0	1	21	As batch 65
	77	6·4	4	2	Contributors consumed no alcohol nor medicines
	78	2·5	2	1	As batch 77
	79	29·0	7*	2	Ether-extractable porphyrins of each individual's contribution $<7{\cdot}5\,\mu g.$/100 ml.* Subjects known to be healthy, but not subject to any restrictions
	80	10·6	Unknown	2	As batch 69
	81	19·4	Unknown	3	As batch 69
Rabbit	62	5·5	4	6	Total 20 rabbit days
	63	12·0	5	12	Total 54 rabbit days
	64	12·8	5	24	Total 113 rabbit days
	67	10·4	4	10	Total 40 rabbit days

* The contributors to this batch included the subject who provided batches no. 65 and 76, and three of the subjects who provided batch 77. There was no other overlap between the known contributors.

batches collected at −20°) no steps were taken to promote, or prevent, conversion of precursors into free porphyrins. The general procedure used for extracting porphyrins is shown in Fig. 1. Procedures indicated by broken lines or enclosed in brackets were used only in certain batches, and are discussed below. More detailed accounts of most of the procedures have already been given (Weatherall, 1952; Comfort & Weatherall, 1953). The quantity of porphyrin examined at one time varied from about 0·2 to 2·0 mg.

Chromatography of porphyrins and porphyrin esters. The methods of Nicholas & Rimington (1949, 1951), Nicholas (1951) and Chu, Green & Chu (1951) were used with the minor modifications already described (Comfort & Weatherall, 1953).

RESULTS

Human urine

On chromatography on aluminium oxide columns, the methyl esters from each batch of talc-extracted urine yielded two, three or occasionally four bands which fluoresced red in ultraviolet light. The separations were less consistent and the concentration of solvent required to elute each band was more variable than we have observed with porphyrins from pathological urines. This was not surprising, as the porphyrins were concentrated from larger volumes of urine and were almost certainly less pure. Similar results were obtained with grade IV Al_2O_3 prepared by us and with a specimen similar to that used by Nicholas & Rimington (1949), kindly provided for comparison by Professor C. Rimington. The first band was the largest, and it was eluted by benzene–chloroform mixtures varying in proportion from 5:1 to 1:5 by vol. Usually it was eluted by 1:1 mixtures, and this, as well as the quantity of material, suggested that it was coproporphyrin. The later fractions were less well separated and are discussed below.

Coproporphyrin fraction. When samples of this fraction were dried, hydrolysed and chromatographed on paper with lutidine and water, they were found to behave mainly or entirely like coproporphyrin (I or III). The unhydrolysed ester on kerosene paper chromatography gave a spot like that of coproporphyrin III, and sometimes other, smaller, unidentified spots. However, no spot behaving like coproporphyrin I was found at this stage.

The coproporphyrin fractions of batches 65, 74, 76 and 77 were combined and dried. After washing with light petroleum and an unsuccessful attempt at crystallization from chloroform, the material soluble in ice-cold anhydrous ether was separated from the remainder. About half the material dissolved readily while the remainder did not go into solution; but on chromatography from kerosene mixtures both fractions gave only one spot, corresponding in position to coproporphyrin III. Chromatography of each of these fractions mixed with an approximately equal quantity of coproporphyrin I gave a good separation in the expected proportions, so that the apparent absence of coproporphyrin I in the experimental material was not simply due to the presence of interfering substances. The fractions were recombined and purified

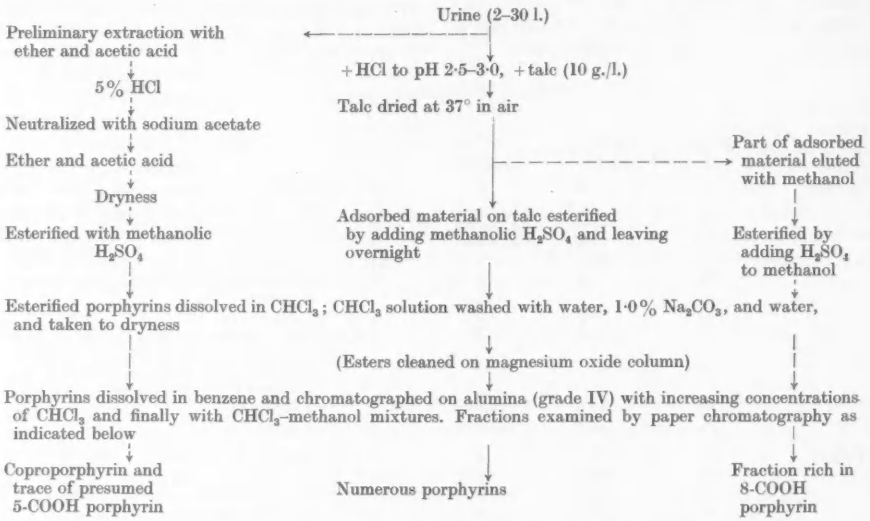
Urine (2–30 l.)
Preliminary extraction with ether and acetic acid
5% HCl
Neutralized with sodium acetate
Ether and acetic acid
Dryness
Esterified with methanolic H_2SO_4
+HCl to pH 2·5–3·0, +talc (10 g./l.)
Talc dried at 37° in air
Part of adsorbed material eluted with methanol
Adsorbed material on talc esterified by adding methanolic H_2SO_4 and leaving overnight
Esterified by adding H_2SO_4 to methanol
Esterified porphyrins dissolved in $CHCl_3$; $CHCl_3$ solution washed with water, 1·0% Na_2CO_3, and water, and taken to dryness
(Esters cleaned on magnesium oxide column)
Porphyrins dissolved in benzene and chromatographed on alumina (grade IV) with increasing concentrations of $CHCl_3$ and finally with $CHCl_3$–methanol mixtures. Fractions examined by paper chromatography as indicated below
Coproporphyrin and trace of presumed 5-COOH porphyrin
Numerous porphyrins
Fraction rich in 8-COOH porphyrin

Fig. 1. Methods of extraction and separation of porphyrins from urine. The steps indicated by broken lines, or enclosed in brackets, were performed only with certain batches, as described in the text.

by hydrolysis in 7 N-HCl for 36 hr., neutralization with NaOH and sodium acetate, and repeated transfer between ether and 0·07 N-HCl. The material was then washed with water, dried and re-esterified. At this stage a trace of coproporphyrin I as well as much of type III was detectable on kerosene chromatography. On crystallization from chloroform–light petroleum a few rosettes of prisms were obtained which melted at 148°, but some microscopically visible non-crystalline material was also present.

Other fractions. The fractions which were eluted from the alumina column after coproporphyrin were smaller and less well defined. With increasing proportions of chloroform in benzene, and then with methanol–chloroform (1:30, v/v), one or more fractions were obtained which yielded on hydrolysis mixtures of porphyrins all with R_F in lutidine–water in the range 0·06–0·76, i.e. in our conditions equal to or between coproporphyrin and uroporphyrin. The largest number of spots in a single sample was six. The frequency distribution of all the observed R_F values is shown in Fig. 2, and their interpretation is discussed below. Many of the fractions were too small for any further investigation. The larger ones were combined and chromatographed again on alumina. A faint trace of red fluorescent material, too small for any examination, was obtained from the column with benzene–chloroform (1:2, v/v), but otherwise no porphyrin was eluted until chloroform–methanol mixtures were used. The biggest fraction was eluted with chloroform–methanol (200:1, v/v), and appeared to contain mainly uroporphyrin (lutidine R_F 0·09, with smaller spots at 0·18 and 0·32). Chloroform–methanol (100:1, v/v) brought down a smaller band with, surprisingly, porphyrins with a much higher R_F in lutidine–water (0·87 and 0·72 and a trace at 0·18; coproporphyrin on this paper = 0·76); and what remained on the column was eluted only with much methanol (1:1, by vol., or more in chloroform) and had a similar composition to the main band (lutidine–water R_F 0·09, 0·18, 0·31 and 0·43). These separations were not altogether satisfactory, but attempts to isolate the fractions on columns of magnesium oxide or of calcium carbonate were less successful, and the amount of material was insufficient for further analysis.

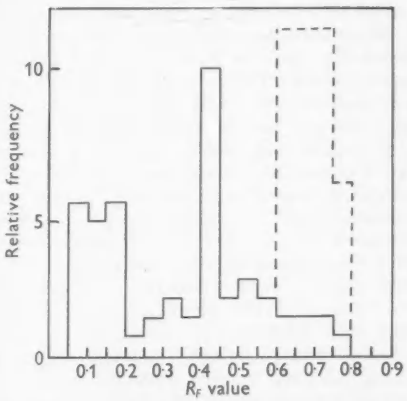

Fig. 2. Frequency distribution of R_F values of spots found on lutidine paper chromatography of hydrolysates of fractions obtained after elution of the main band of porphyrin esters from Al_2O_3 columns. The range of values observed with coproporphyrin fractions is shown in broken outline: the total proportion in the starting material was considerably greater than that indicated.

Other methods of separation. Further evidence was obtained about the minor fractions by extracting half of one 10 l. batch of urine (no. 69) with acetic acid and ether before adsorbing any remaining porphyrins on talc.

All the porphyrins were esterified and chromatographed on aluminium oxide. The ether extract yielded almost entirely coproporphyrin ($R_F = 0·64$) with a little porphyrin with a lower R_F (0·52) in lutidine–water. The residue after ether extraction contained a trace of coproporphyrin ($R_F = 0·68$) and a mixture of porphyrins with lower R_F (0·50, 0·37, 0·24 and 0·12) which were not separated on the column. The other half of the batch of urine was treated with talc without preliminary extraction, and was found to contain porphyrins corresponding to the sum of the two fractions, i.e. mainly coproporphyrin and a group of porphyrins with lower R_F values.

Towards the end of these investigations, two batches of urine were treated with talc as usual, and the talc was then washed with methanol before adding the esterifying mixture of methanol and sulphuric acid. This washing was intended to remove some of the non-porphyrin pigments and provide cleaner material for the alumina columns. However, the methanol eluted some porphyrin, which on esterification and purification on aluminium oxide columns appeared to contain a lower proportion of coproporphyrin than usual. Subsequent treatment of the talc with methanolic sulphuric acid yielded mainly coproporphyrin. This observation was not pursued further, but might offer a more satisfactory method of concentrating intermediate fractions.

Attempts to isolate the normal urinary porphyrins by adsorption on calcium phosphate precipitated from the urine by adding calcium chloride and sodium hydroxide (Dobriner, 1936; Sveinsson, Rimington & Barnes, 1949) were not successful, and in view of the good results with talc, this method was not pursued.

Other pigments. A number of other pigments appeared on the alumina column as esters. These included a bright-blue, non-fluorescent pigment eluted next after coproporphyrin ($\lambda_{max.} = 565$ mμ. in $CHCl_3$), and a substance with the colour of permanganate closely similar to the porphyrin-accompanying pigments found by one of us in marine molluscs (Comfort, 1949). A considerable amount of brownish pigments always remained at the column head, and green or yellow bands, possibly pyrrolic, came down between or with the porphyrins in several batches. None of these pigments was recovered in crystallizable amounts.

Rabbit urine

Talc extracts of rabbit urine contained a considerably larger amount of non-fluorescent pigments relative to their porphyrin content than did extracts of human urine. The most satisfactory solid chromatograms were obtained after preliminary purification of the esters in chloroform (B.P.) on a magnesium oxide column, or on an aluminium oxide column which was developed first with benzene (which removed some greenish pigments but no porphyrin) and then with chloroform (B.P.) (which removed practically all of it). Ethereal extracts of rabbit urine were cleaner, and the esterified material from these was run directly on aluminium oxide.

The fractions obtained from aluminium oxide chromatography of rabbit urine were different from those obtained from human urine. At least one porphyrin was present which could be eluted more easily than coproporphyrin: it was eluted consistently by benzene–chloroform (approx. 10:1, v/v), and on hydrolysis and lutidine paper chromatography it had an R_F suggesting a dicarboxylic porphyrin. On kerosene paper chromatography its R_F in both solvent systems was high and suggested its similarity to protoporphyrin, and also to the porphyrin *A* found in the urine of lead-poisoned rabbits (Comfort & Weatherall, 1953).

The main band on aluminium oxide columns behaved like coproporphyrin. On kerosene paper chromatograms it appeared to consist almost entirely of coproporphyrin III, with a minute trace of coproporphyrin I. The remainder of the material eluted from the columns contained mixtures of porphyrins and often other pigments. A zone of green pigment normally appeared in the region between porphyrin *A* and the coproporphyrin band, and hid any tricarboxylic porphyrin which might have been there; but traces of tricarboxylic porphyrin were sometimes detected in lutidine paper chromatograms of the eluate from this zone. Porphyrins with R_F intermediate between copro- and uro-porphyrin, but closer to that of coproporphyrin, were found by lutidine chromatography of hydrolysates of the tail fractions from the column, and as traces in the main coproporphyrin band. Both the greenish pigment and a yellow fluorescent oil with a strong odour appeared as persistent contaminants in these fractions, even after repeated chromatography.

DISCUSSION

These results show the existence in the urine of healthy humans and rabbits of traces of several porphyrins besides coproporphyrins I and III. These porphyrins in some respects at least resemble those found in porphyric urine (Macgregor, Nicholas & Rimington, 1952; Rimington & Miles, 1951) and in the urine of lead-poisoned rabbits (Comfort & Weatherall, 1953). The quantities involved are small; at most, all the minor fractions together amount to less than 10–20% of the total, and their combined excretion is therefore probably less than 5–10 μg./man/day. Their identification in human urine rests on their inextractibility from urine by ether (with an exception mentioned below), the behaviour of their esters on aluminium oxide columns, and particularly on their R_F value using lutidine paper chromatography. Most of them have properties intermediate between those of coproporphyrin and uroporphyrin, and it seems likely that they contain five, six or seven carboxyl groups. The simple linear relation between the number of carboxyl groups and the R_F in lutidine–water, postulated by Nicholas & Rimington (1949), cannot be applied rigidly to our material, because we have sometimes observed as many as five spots with R_F values lower than that of coproporphyrin, and because the spots we have found are not always regularly spaced. The frequency distribution of the spots shows a maximum midway between coproporphyrin and uroporphyrin; material giving a single spot in this region has been isolated and shown also to behave homogeneously on kerosene chromatography and it is provisionally assumed to be a hexacarboxylic porphyrin. There are also suggestions of smaller peaks in the frequency distribution at sites suggesting penta- and hepta-carboxylic porphyrins. As we have found a spot corresponding to a pentacarboxylic porphyrin (R_F in lutidine–water about 0·52) in ether extracts of both human and rabbit urine, without the presence of any slower running fraction, it seems probable that this also represents a separate entity. The remaining spots may include one due to a heptacarboxylic porphyrin, or be due to two isomers with the same number of carboxyl groups (cf. Falk, 1954), or be due to impurities which we have failed to eliminate from the material and which have caused splitting of homogeneous porphyrin spots. In any case, proper identification of these intermediate porphyrins is

not feasible until adequately identified standard materials are available for comparison.

The porphyrins obtained from normal rabbits appear to differ from those found in human urine. Extraction with talc has not been satisfactory for the concentration of porphyrin from large amounts of rabbit urine, owing to the heavy contamination of the product with other pigments. In view of this difficulty, the failure to find uroporphyrin may not be significant. However, the porphyrins recovered from talc appeared identical with, though less pure than, those obtained by ether extraction. They consisted chiefly of coproporphyrin with small admixture of porphyrins of lower and higher carboxyl numbers, ranging from di- and tri- to penta- and possible hexa-carboxyl porphyrins, in contrast to the human range, which appeared to extend from tetra- or possibly tri-carboxyl to octa-carboxyl porphyrins.

The isomer distribution of the human coproporphyrin was unexpected. The widespread view that coproporphyrin I regularly predominates in normal urine is not, however, very well founded in experimental evidence. Type I coproporphyrin has been identified by the melting point of its crystals by Fink & Hoerburger (1934), Watson (1936) and Grotepass (1938). However, Watson's material melted at a temperature as low as 218–223°, and also contained some prisms which looked like the type III ester; and Grotepass crystallized almost as much type III ester as type I. The objection that Grotepass's material may have originated partly from abnormal subjects (Dobriner & Rhoads, 1940; Watson & Larson, 1947) is purely hypothetical and appears to be no more than a convenient way of dismissing an inconvenient finding. In a more recent study (Watson, Hawkinson, Schwartz & Sutherland, 1949), by the fluorescence quenching method (Schwartz, Hawkinson, Cohen & Watson, 1947), groups of normal subjects have been found in all of whom type I coproporphyrin predominated and other groups in whom type III was the main constituent. In the small groups we have examined, type III coproporphyrin always predominated, and it was only after considerable pooling of material and purification that type I could be found chromatographically at all. In one group the coproporphyrin concentration of each individual's urine was measured and found not to exceed 7·5 μg./100 ml.; the possibility of a contributor with idiopathic type III coproporphyrinuria (Watson, Schwartz, Schulze, Jacobson & Zagaria, 1949) is therefore excluded: nor did any of the other batches of urine yield notably more porphyrin esters in relation to their size than did this one. In any case there would have to be a surprisingly large number of such 'abnormal' subjects in order to affect all the groups we examined.

It seems much more probable that the normal urinary isomer type is as variable as the evidence suggests, and that it is undesirable to postulate that either type 'normally' predominates. Since a large part or perhaps practically all of the urinary coproporphyrin is formed from a precursor or precursors after the urine has been secreted by the kidneys (Watson, de Mello, Schwartz, Hawkinson & Bossenmaier, 1951; Eriksen, 1952; Weatherall, 1952), the isomer type may depend on the kind of precursor, or it may depend on the reaction by which the precursor is converted into the free porphyrin. If the precursors are of the tetrapyrrolic porphyrinogen type, as the conversion by iodine suggests (Watson *et al.* 1951), change of isomer type is rather unlikely during the reaction. However, the precursors present in urine are not necessarily always or only of this type, and if any substantial portion of the precursor material is a monopyrrole similar to porphobilinogen (Cookson, Rimington & Kennard, 1953), the final isomer proportion may be much influenced by minor physical or chemical differences in the urinary composition or in the method of treating the urine. It has recently been found (Cookson & Rimington, 1954) that the proportions of uroporphyrin isomers formed from porphobilinogen depend on the pH of the solution, and it may therefore be of significance that Watson and his colleagues (Schwartz, Zieve & Watson, 1951; Watson *et al.* 1951) have been working with urine kept alkaline with sodium carbonate during the period of collection. It is possible that the subsidiary fractions which we and others have described are also formed to a variable extent after the urine has been secreted, and until more is known about the identity of the precursors, caution is necessary in attaching much significance to the individual porphyrins recoverable from mammalian urine.

SUMMARY

1. The porphyrins present in the urine of normal men and rabbits have been examined chromatographically.

2. Human urine contained coproporphyrin, a little uroporphyrin, and small amounts of porphyrins with, probably, five, six and seven carboxyl groups.

3. Rabbit urine contained coproporphyrin and traces of other porphyrins with, probably, between two and six carboxyl groups.

4. The coproporphyrin was in both species predominantly of type III. Statements that the normal human urinary coproporphyrin is of type I are discussed in the light of this finding.

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Studies on Sulphatases

8. THE ARYLSULPHATASE OF A STRAIN OF *ALCALIGENES METALCALIGENES* ISOLATED FROM INTER-TIDAL MUD

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Karunairatnam & Levvy (1951) showed that the β-glucuronidase activity found in sheep rumen was due to micro-organisms, and Marsh, Alexander & Levvy (1952) suggested that the high β-glucuronidase activity found in the omasum, rumen, caecum and colon of many animals might have a similar origin. It was possible that the high arylsulphatase activity of the visceral humps of marine molluscs (Dodgson, Lewis & Spencer, 1953) was due to the presence of micro-organisms, although arylsulphatase activity in bacteria is not common. Barber, Brooksbank & Kuper (1951) found activity in only two of a large number of strains of *Staphylococcus pyogenes*. Whitehead, Morrison & Young (1952) detected the enzyme in certain salmonellae and mycobacteria after studying 212 strains from a wide range of bacterial species, while Hare, Wildy, Billet & Twort (1952) found activity in only two out of ninety-nine strains of anaerobic cocci. Harada (1948) noted arylsulphatase activity in strains of *Streptococcus alcalophilus* isolated from urine and soil.

We have been unable to detect any significant arysulphatase activity in bacteria isolated from the visceral humps of a number of different marine molluscs but have noted appreciable enzyme activity in two micro-organisms isolated from certain inter-tidal muds. The least active of these organisms, a yeast identified as *Trichosporon cutaneum*, has not been further investigated but the other, a bacterium of the genus *Alcaligenes*, is interesting, since it is an example of an arylsulphatase with an optimum pH well on the alkaline side of neutrality. A preliminary account of this work has already been given (Dodgson, Melville, Spencer & Williams, 1954).

MATERIALS AND METHODS

Arylsulphatase substrates. The following compounds were prepared: potassium *p*-acetylphenyl sulphate (Dodgson & Spencer, 1953), tripotassium phenolphthalein disulphate (Whitehead *et al.* 1952); potassium *p*-nitrophenyl sulphate (Burkhardt & Lapworth, 1926) and dipotassium 2-hydroxy-5-nitrophenyl sulphate (nitrocatechol sulphate, Roy, 1953*a*).

Detection of arylsulphatase activity. Barber *et al.* (1951) studied arylsulphatase activity in bacteria by detection of phenolphthalein liberated from phenolphthalein disulphate which was incorporated in the medium. This principle was used in the present work but the organisms were grown on solid media, either nutrient agar (1% (w/v) 'Difco' peptone (Difco Laboratories Inc., Detroit, U.S.A.), 0·3% (w/v) 'Difco' beef extract, 0·5% (w/v) NaCl and 1·5% (w/v) 'Difco' agar) or sea-water agar (Zobell, 1946). Arylsulphatase-producing colonies could be subcultured without difficulty after detection by exposure to NH_3 providing subculturing took place at once, but re-incubation of plates subsequent to treatment with NH_3 led to the destruction of the organisms (cf. Bray & King, 1943). The method was tested using the known arylsulphatase producers, *Mycobacterium piscium* and *Myco. ranae* (Whitehead *et al.* 1952).

Determination of arylsulphatase activity of growing cultures. The method of Whitehead *et al.* (1952) was modified. Centrifuge tubes graduated at 7 ml. were dried at 105°, weighed and 0·7 ml. of 0·01 M tripotassium phenolphthalein disulphate was added to each tube. Control tubes containing 0·7 ml. water were prepared. The tubes were plugged with cotton wool and autoclaved for 20 min. at 20 lb./sq.in., no destruction of substrate occurring under these conditions. Sterile nutrient broth (1% (w/v) 'Difco' peptone, 0·3% (w/v) 'Difco' beef extract and 0·5% (w/v) NaCl) was added to each tube to give a total volume of 7 ml. and after incubation overnight at 37·5° any tubes showing signs of growth were rejected. The remaining tubes were inoculated with 2 drops of a culture of the organism and incubated at 25 and 37·5°. Tubes were tested at intervals of 2, 4, 8 and 16 days as follows: the volume of liquid in each tube was adjusted to 7 ml. with water to compensate for loss due to evaporation, and the bacteria were separated by centrifuging. A portion (5 ml.) of each clear supernatant was mixed with 5 ml. of glycine buffer, pH 10·8 (Talalay, Fishman & Huggins, 1946) and the red phenolphthalein colour measured spectrophotometrically (Hilger Uvispek) at 560 mμ. using the control tubes as blanks. A calibration curve was prepared using varying concentrations of phenolphthalein in sterile medium. The bacterial cells were washed with water, centrifuged and finally dried overnight at 105° for dry-weight determination. It was possible that the organisms attacked the liberated phenolphthalein; to check this, tubes containing 0·7 ml. of a solution of phenolphthalein (100 μg./ml. in 10% (v/v) ethanol) were treated as above and the amount of phenolphthalein remaining at the end of 16 days was measured.

Determination of arylsulphatase activity of bacterial preparations. The problems arising from the use of phenolphthalein diphosphate in studying the kinetics of phosphatases have been outlined by Huggins & Talalay (1945). Similar considerations apply to the use of phenolphthalein disulphate as a substrate for arylsulphatase and for this reason other substrates were used for studying the properties of the bacterial enzyme. When potassium *p*-acetylphenyl sulphate was used as substrate, enzyme activity was measured by the method of Dodgson, Spencer & Thomas (1953) while in experiments with potassium *p*-nitrophenyl sulphate the liberated *p*-nitrophenol was determined in substantially the same way, the final spectrophotometric measurement being made at 400 mμ. (Dodgson & Spencer, 1953). When the substrate was nitrocatechol sulphate the method of Roy (1953*a*) occasionally failed to give satisfactory duplicate determinations and was modified by omitting the precipitation of protein with phosphotungstic acid. The incubation mixture contained 0·2 ml. buffered substrate and 0·2 ml. buffered enzyme. After incubation, 2 ml. of the alkaline quinol mixture was added and, after centrifuging, the intensity of the red colour produced was measured at 515 mμ., the wavelength of maximum absorption of nitrocatechol under these conditions ($E_{max.}$ 11300). In all cases sodium salts were used in preparing the buffers.

Nitrogen estimation was by the micro-Kjeldahl method (Markham, 1942).

RESULTS

Examination of mollusc suspensions for arylsulphatase-producing bacteria. Molluscs were collected locally. The visceral humps were dissected out, washed in sterile sea-water and suspended in the same medium using a sterile glass homogenizer (Potter & Elvehjem, 1936) under aseptic conditions to give a 10% (wet wt./v) suspension. A loose-fitting pestle was used in the homogenizer and the time was limited to 1 min. in order to break up the tissues without appreciably destroying the bacteria present. Plates prepared as described earlier were streaked with these undiluted suspensions and then incubated at 25° under both aerobic and anaerobic conditions. Good growth occurred with both ordinary and sea-water (Zobell, 1946) nutrient media under aerobic conditions but anaerobic growth was poor. In general, no arylsulphatase-producing organisms were isolated from molluscs collected in various localities on the Bristol Channel. Occasionally weak arylsulphatase activity was detected but this invariably disappeared on subculturing and was probably due to the molluscan arylsulphatase present in the initial streaks. Enrichment of the culture media by addition of mollusc suspensions in which arylsulphatase activity had been destroyed either by boiling or by incubation of sterilized filtrates for 3 weeks at 37·5° (see Dodgson, Lewis & Spencer, 1953) did not result in the isolation of arylsulphatase-producing organisms.

Arylsulphatase producers in inter-tidal mud. Other possible marine sources of arylsulphatase-producing bacteria were examined under aerobic and anaerobic conditions using nutrient agar and sea-water agar plates. Agar-digesting organisms isolated according to Stanier (1941) from the red seaweed *Corallina* did not attack phenolphthalein disulphate. Samples of undiluted inter-tidal mud collected from Sully, Glamorgan, gave organisms with arylsulphatase activity when grown aerobically on both nutrient and sea-water agar, although some colonies lost their activities after subculturing. Anaerobic growth was poor. Occasional activity was found in cultures from mud collected from Barry harbour but in general, mud samples from a number of other areas of the Bristol Channel

Table 1. *The hydrolysis of phenolphthalein disulphate by certain micro-organisms*

Growth under the conditions outlined in the text for 16 days. In the recovery controls, organisms were grown without phenolphthalein disulphate but in the presence of 70 μg. phenolphthalein.

Organism	Temperature of growth (°)	Total phenolphthalein liberated (μg.)	Phenolphthalein liberated/mg. dry wt. of organism (μg.)	Recovery of added phenolphthalein (%)
Myco. piscium	37·5	237	39	95
	25·0	114	12	98
Myco. ranae	37·5	31	4	92
Alcaligenes metalcaligenes	25·0	395	72	83
Trichosporon cutaneum	25·0	38	6	82

revealed no arylsulphatase producers. Arylsulphatase-producing organisms from Sully mud grown on nutrient agar were repeatedly subcultured on this medium and two aerobic micro-organisms capable of attacking arylsulphates were isolated and identified. One of these organisms was a yeast, *Trichosporon cutaneum*, the other a bacterium of the genus *Alcaligenes*. Bacteriological identification tests (Bergey, 1948) showed the bacterium to be a strain of *A. metalcaligenes*. Attempts were made to estimate the arylsulphatase activity of these organisms but the results were obscured by the fact that both were capable of destroying phenolphthalein. However, the enzyme activity of *Trichosporon* was comparable to that of *Myco. ranae* while that of *Alcaligenes* was greater than that of *Myco. piscium* (Table 1).

Table 2. *Distribution of arylsulphatase activity in cultures of* Alcaligenes metalcaligenes *and in extracts obtained after partial destruction of the cells in a bacterial mill*

The cells from 1·6 l. of the bacterial culture were suspended in 40 ml. distilled water for crushing. Enzyme activities were measured at 37·5° over a period of 18 hr. in the presence of 0·006 M potassium *p*-acetylphenyl sulphate in 0·5 M sodium acetate at pH 6·5. Results are the average of two experiments.

Fraction (from 1·6 l. of bacterial culture)	Enzyme activity (μg. *p*-hydroxyacetophenone liberated)	
	Whole fraction	Per mg. N
Culture fluid	1670	0·62
Cells	4352	53
Supernatant from crushed cells	2300	90
Debris from crushed cells	2514	45

Preparation of an enzyme concentrate from Alcaligenes. Attempts were first made to extract the enzyme from the bacterial cell. The organism was grown aerobically in the nutrient broth at room temperature or 25° for 7 days, when the cells were spun down at 0°, washed with a little ice-cold water and the washings added to the supernatant. Measurement of arylsulphatase activity of the supernatant using potassium *p*-acetylphenylsulphate as substrate showed that about one-third of the total activity was associated with the cell-free culture fluid. The bacterial mass was suspended in ice-cold distilled water and ground for 2 hr. in a bacterial mill (Booth & Green, 1938), during which time the temperature of the suspension increased to 10°. The resultant suspension was centrifuged at 0° and the total arylsulphatase activity and the activities of the debris and supernatant were measured (Table 2). The bacterial mill released only about 50% of the enzyme of the cell into solution. Acetone was more effective in rupturing the bacterial cells and an acetone-dried preparation of the bacterial mass retained about 90% of the original arylsulphatase activity, 70% of the activity of the powder being soluble in water.

Table 3. *The yields and activities of concentrates of the arylsulphatase of* Alcaligenes metalcaligenes

The powders were suspended in a suitable volume of 0·1 M sodium phosphate, pH 8·75 and enzyme activities were measured over 1 hr. using 0·4 ml. enzyme suspension and 0·4 ml. 0·006 M potassium *p*-acetylphenyl sulphate dissolved in the same buffer. The results are the average of ten 16 l. culture experiments.

Fraction	Yield (g.)	Arylsulphatase activity (μg. *p*-hydroxyacetophenone liberated)	
		Whole fraction	Per mg. N
Whole culture (16 l.)	—	353 000*	—
Powder *A*	5	210 000	370
Powder *B*	0·67	108 540	1190

* Measured over 18 hr. in 0·1 M sodium phosphate, pH 8·75.

On a larger scale, *Alcaligenes* was grown aerobically in large flasks each containing 16 l. of the nutrient broth, the pH being adjusted to 7·4–7·6 with N-NaOH. The cultures were aerated by means of a sterile air-stream and incubated at room temperature or at 25°. After 6 days the cells were spun down at 0°, washed with ice-cold water and again separated at 0°. No attempt was made to recover that part of the enzyme activity which was associated with the culture fluid in view of the low enzyme concentration in terms of nitrogen content (Table 2). The cells were suspended in ice-cold acetone using a Townson & Mercer macerator, filtered at the pump and washed well with ice-cold acetone. After drying *in vacuo*, a stable light-coloured powder remained (powder *A*), the average yield and activity of which is recorded in Table 3. The powder (5 g.) was suspended in ice-cold water (75 ml.) using the Townson & Mercer macerator and allowed to stand at 0° for 90 min. The

suspension was clarified by centrifuging at 0° (24000 *g*) and the debris resuspended in 60 ml. of ice-cold water. After centrifuging, the two clear supernatants were combined and treated with ice-cold acetone (Askonas, 1951) until the concentration of acetone was 85% (v/v). The resulting precipitate was separated at the pump, washed well with ice-cold acetone and dried *in vacuo* (powder *B*). The yield and activity of this powder is recorded in Table 3. The concentrate is stable when stored in a stoppered bottle at 0°.

Properties of the enzyme concentrate. When the arylsulphatase activity was measured over 1 hr. at 37·5° with potassium *p*-acetylphenyl sulphate (0·003 M) as the substrate, the optimum pH of the enzyme in 0·1 M phosphate was 8·75 (Fig. 1). This high optimum pH was not an artifact arising from the acetone treatment since an aqueous extract of the bacteria prepared by crushing the cells in a bacterial mill (Booth & Green, 1938) showed the same optimum pH. Maximum activity of the powder occurred at a substrate concentration of 0·003 M in 0·1 M phosphate at pH 8·75, the enzyme being inhibited by excess substrate (Fig. 2). Using these optimum conditions the effects of enzyme concentration, time of incubation and time of incubation before the addition of substrate, on the activity of the enzyme were studied (Figs. 3 and 4). The enzyme was rapidly inactivated at temperatures greater than 50° and at pH lower than 5.

Using potassium *p*-nitrophenyl sulphate (0·0015 M in 0·1 M phosphate) maximum activity was again obtained at pH 8·75 (Fig. 1). At this pH the optimum substrate concentration was 0·0015 M

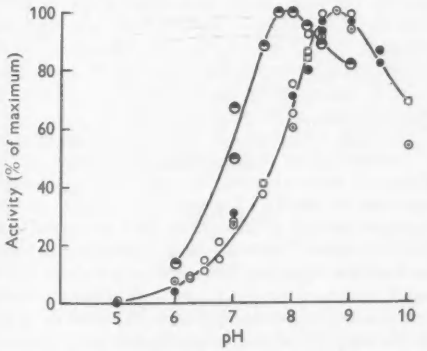

Fig. 1. pH/activity curves for the arylsulphatase of an acetone-dried concentrate of *A. metalcaligenes* (powder *B*) using different substrates in the presence of 0·1 M phosphate buffer. The concentration of the enzyme solution was 0·1% (w/v) when nitrocatechol sulphate was the substrate and 0·05% with other substrates. ◓—◓, nitrocatechol sulphate (0·015 M); ●—●, *p*-nitrophenyl sulphate (0·0015 M); ○—○, *p*-acetylphenyl sulphate (0·003 M); ⊙—⊙, *p*-acetylphenyl sulphate (0·003 M) acted upon by an aqueous extract of the crushed bacteria (see text). Incubation was for 1 hr. at 37·5°.

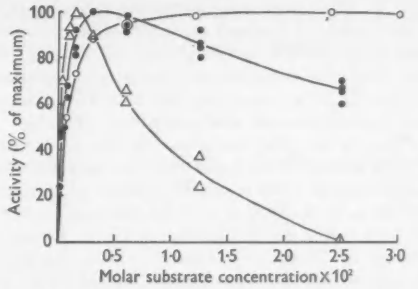

Fig. 2. Substrate concentration/activity curves for the arylsulphatase of *A. metalcaligenes* (powder *B*) acting on different substrates in the presence of 0·1 M phosphate buffer adjusted to the appropriate optimum pH. Incubation was for 1 hr. at 37·5°. The concentration of the enzyme solution was 0·1% (w/v) when nitrocatechol sulphate was the substrate and 0·025% with other substrates. ○—○, nitrocatechol sulphate (pH 8·0); ●—●, *p*-acetylphenyl sulphate (pH 8·75); △—△, *p*-nitrophenyl sulphate (pH 8·75).

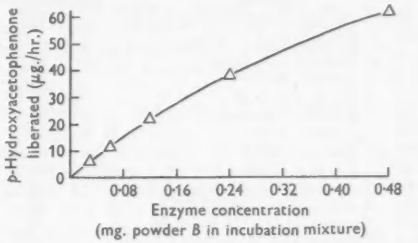

Fig. 3. Enzyme concentration/activity curve for the arylsulphatase of *A. metalcaligenes* (powder *B*) acting on 0·003 M potassium *p*-acetylphenyl sulphate in 0·1 M phosphate, pH 8·75 at 37·5° for 1 hr.

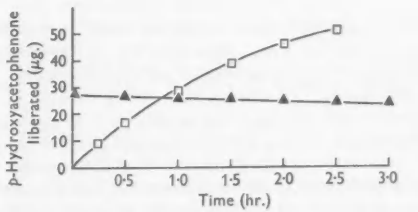

Fig. 4. Time/activity and pre-incubation/activity curves for the arylsulphatase of *A. metalcaligenes* acting on 0·003 M *p*-acetylphenyl sulphate in 0·1 M phosphate, pH 8·75. Each incubation mixture contained 0·18 mg. of powder *B*. In the pre-incubation–activity experiment the enzyme preparation was incubated at 37·5° for varying periods before addition of substrate and was subsequently incubated for 1 hr. at 37·5°. □—□, time/activity; ▲—▲, pre-incubation/activity.

(Fig. 2). With increasing substrate concentration, enzyme activity declined rapidly until at a concentration of 0·025M activity was nil. Using the optimum concentration of nitrocatechol sulphate (0·015M in 0·1M phosphate, see Fig. 2) maximum activity was obtained at about pH 8·0 (Fig. 1).

Presence of other enzymes in the concentrate. Powder *B* showed no β-glucuronidase activity when tested against 0·004M *p*-chlorophenyl glucuronide (Dodgson *et al.* 1953*a*) in 0·1M phosphate at pH values 6–9 and in 0·5M acetate at pH 6. Using the method of Dodgson & Spencer (1954) no glycosulphatase activity was observed at pH values 5–8 towards 0·005M potassium glucose 6-sulphate in 0·5M acetate.

Table 4. *Recovery of added phenolphthalein from growing cultures of various* Alcaligenes *organisms*

Organisms obtained from the National Collection of Type Cultures were grown at 25° for 7 days under the conditions outlined in the text.

Organism (National Type Culture designation)		Recovery of added phenolphthalein (%)
6535	*Alcaligenes bookeri*, Beef	60
712	*A. faecalis*, Castellani	48
415	*A. faecalis*, 439	98
1347	*A. faecalis*, Bedson	100
6572	*A. faecalis*, Edwards II	94
8763	*A. faecalis*, 2792	80
8769	*A. faecalis*, 15204	60
8840*	*A. faecalis*, 43024	100
5043	*A. haemolysans*, Baumann	34
3233	*A. subviscosum*, ATCC 337	40
7249	*Alcaligenes* sp. F/16 Freeman	26
7570	*Alcaligenes* sp. Wheatley	36
7991	*Alcaligenes* sp. 21919	6
7992	*Alcaligenes* sp. 21445	6
7993	*Alcaligenes* sp. 21447R	32

* This organism showed very feeble arylsulphatase activity towards phenolphthalein disulphate.

The arylsulphatase activity of related organisms. Other species were examined for arylsulphatase activity. One strain each of *Alcaligenes bookeri*, *A. haemolysans*, *A. subviscosum*, seven strains of *A. faecalis* and five unidentified species were examined but only one strain showed any activity. Recovery of phenolphthalein from growing cultures of the majority of the organisms was poor (Table 4), and it was possible that arylsulphatase activity was being masked by the ability of the organism to destroy the liberated phenolphthalein. However, acetone-dried preparations of two of these organisms (nos. 712 and 7991, see Table 4) showed no arylsulphatase activity at pH values 5, 7 and 9 in 0·1M phosphate using 0·001M tripotassium phenolphthalein disulphate or 0·003M potassium *p*-acetylphenyl sulphate as substrates, although neither acetone-dried preparation was able to metabolize phenolphthalein. The one organism (no. 1347) which did have arylsulphatase activity did not destroy phenolphthalein.

DISCUSSION

It seems clear that the bacteria present in the digestive organs of marine molluscs contribute little or nothing towards the high arylsulphatase activity which is found in these organs. Indeed the present work emphasizes the findings of earlier workers that arylsulphatase activity is not common in bacteria; a point which contrasts sharply with the widespread occurrence of the enzyme in the animal world. However, Whitehead *et al.* (1952) have pointed out the difficulties involved in the detection of arylsulphatase in micro-organisms which are capable of metabolizing phenolphthalein, and the present findings show the need for suitable recovery control experiments in such investigations.

There appears to be no relationship between the various types of organisms which possess arylsulphatase activity, although it is interesting that the two most active organisms examined to date, *Myco. piscium* and *A. metalcaligenes*, were both isolated from a marine environment. However, the high activities observed in these bacteria are somewhat artificial, since enzyme activities were measured at a temperature much higher than that of their normal habitat, and the enzyme from *Alcaligenes* is about 4 times more active at 37·5° than at 10° when measured over a period of 1 hr. *Myco. piscium* liberates more phenolphthalein at 37·5° than at 25° (Table 1), despite the fact that the organism grows better at 25°. The difference may be accounted for by the increased activity of the enzyme at 37·5° rather than an increased production of arylsulphatase at this temperature.

The high optimum pH value of the enzyme of *A. metalcaligenes* may possibly be related to the ability of this organism to produce considerable amounts of alkali. During the growth of the organism the pH of the culture fluid increased from 7·4–7·6 to 8·4–8·7 in 6 or 7 days. The inter-tidal mud in which the organism flourishes has a pH of 8·1–8·2, which is higher than the normal pH of the sea-water in this area (7·5). A comparison of the optimum pH of the enzyme of *A. metalcaligenes* with those of arylsulphatases from other sources is not strictly valid, since these have been mainly studied in the presence of acetate rather than phosphate buffers. Such evidence as is available indicates that in general the optimum pH of the various arylsulphatases is higher in the presence of phosphate (Table 5). The *Alcaligenes* enzyme has optimum activity against nitrocatechol sulphate at a pH lower than that for the other substrates tested; this is in agreement with the findings for arylsulphatases

Table 5. *The* pH *optima of arylsulphatases of different origin*

Optimum concentrations of the sulphates indicated were used in the presence of 0·5 M acetate or 0·2 M phosphate buffers unless otherwise stated.

	Optimum pH using					
	p-Acetylphenyl sulphate		*p*-Nitrophenyl sulphate		Nitrocatechol sulphate	
Source of enzyme	Acetate	Phosphate	Acetate	Phosphate	Acetate	Phosphate
Alcaligenes metalcaligenes	—	8·75*	—	8·75*	—	8·0*
Aspergillus oryzae	6·3	7·6	6·3	—	5·8	—
Rat liver	7·2	8·2	—	—	6·0	—
Human liver	6·9	7·3	7·3	—	6·0	—

* In 0·1 M phosphate buffer.

from other sources which have been studied in this laboratory (Table 5).

Recent investigation of arylsulphatase activity in the livers of rat, ox, mouse, rabbit and man in this laboratory (Dodgson, Spencer & Thomas, 1954; cf. Roy, 1953*a*, *b*) has shown the presence in these tissues of at least two arylsulphatases, which were distinguished by their widely different affinities for nitrocatechol sulphate on the one hand and potassium *p*-acetylphenyl sulphate and potassium *p*-nitrophenyl sulphate on the other. It was possible that two similar enzymes were present in the concentrates from *A. metalcaligenes* but the ratio of activities towards the three substrates under the respective optimum conditions was the same for the culture fluid and powders *A* and *B*. The activity towards nitrocatechol sulphate was about one-fifth of the activity towards the other two substrates when measured on a molar basis.

The importance of arylsulphatase to *A. metalcaligenes* is obscure. The organism grows well in absence of arylsulphate and the enzyme activity is in no way diminished. An attempt has been made to detect spectroscopically the presence of natural substrates of the enzyme in the inter-tidal mud in which the organism flourishes but results obtained so far have been inconclusive.

SUMMARY

1. The high arylsulphatase activity of the visceral humps of marine molluscs is not due to micro-organisms.

2. A strain of *Alcaligenes metalcaligenes* isolated from inter-tidal mud possesses considerable arylsulphatase activity but is free from glycosulphatase and β-glucuronidase.

3. A concentrate of the *Alcaligenes* enzyme has been prepared and some of its properties are described.

4. A number of other bacteria of the genus *Alcaligenes* showed no arylsulphatase activity when tested against phenolphthalein disulphate.

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The Reaction of Metmyoglobin with Strong Oxidizing agents

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In our previous papers (George & Irvine, 1951, 1952, 1953*a*) we showed that the intermediate compound, which is formed when metmyoglobin (MetMb) reacts with hydrogen peroxide, methyl or ethyl hydroperoxide, may be considered as one in which the iron has an effective oxidation number of +4. Its relationship to MetMb, represented by $Fe_p^{3+}(H_2O)$, is given by the equation,

$$Fe_p^{3+}(H_2O) \rightleftharpoons Fe_p^{IV} + e^-,$$

where Fe_p^{IV} stands for the intermediate compound. Since the reaction of MetMb with peroxides is an irreversible, single equivalent oxidation, rather than an equilibrium reaction of the type envisaged in the formation of an enzyme–substrate complex, it seemed possible that other oxidizing agents would also be capable of forming the compound.

Taube & Cahill (1951) showed that tetrasulphonated phthalocyanines (TSCP) can be oxidized by electron removal with the ceric ion

$$TSCP \rightarrow TSCP^+ + e^-, \qquad (1)$$

and so it was of interest to see whether electron transfer oxidizing agents would be effective in the case of the structurally similar MetMb. Reactions of MetMb with such oxidizing agents ought to be free from the complications inherent in its reactions with the peroxides. Of the two oxidizing equivalents of peroxides, one is transferred to MetMb while the second appears as a transient oxidizing entity. This entity has recently been shown to be involved in competitive reactions, some of which involve attack on the porphyrin ring (George & Irvine, 1953*a*).

Our attention has recently been drawn to the papers of Polonovski, Jayle, Glotz and Fraudet, published between 1938 and 1941 (full references are given later), who suggested, as we have done more recently, that quadrivalent iron compounds participate in the methaemoglobin and peroxidase reactions with peroxides. Since their publication, these papers have escaped notice in most discussions of the reaction mechanism, so we have briefly reviewed the experimental evidence, and the detailed mechanism proposed, at the end of this paper.

EXPERIMENTAL

Potassium molybdocyanide ($K_4Mo(CN)_8$). This was prepared according to Willard & Thielke (1935). After three recrystallizations, titration with $KMnO_4$ showed the sample to be 99% pure; stock solutions were therefore made up by weight. Experiments with $K_4Mo(CN)_8$ were carried out in the dark because of the extreme sensitivity to light of the $K_3Mo(CN)_8$ produced by its oxidation.

Potassium molybdicyanide ($K_3Mo(CN)_8$). This was prepared according to Willard & Thielke (1935). Because it hydrolyses rapidly in the presence of light, both its preparation and the experiments with MetMb were carried out in the dark.

Potassium chloriridate (K_2IrCl_6). This was obtained from Messrs Johnson, Matthey and Co. Ltd. Fresh solutions were prepared immediately before use and standardized by spectrophotometric titration with AnalaR $K_4Fe(CN)_6$. This precaution was necessary because of the slow hydrolysis of K_2IrCl_6 in aqueous solution.

Ruthenous tris-dipyridyl chloride ($Ru(dipy)_3Cl_2$, $6H_2O$). This was prepared from ruthenium trichloride and αα′-dipyridyl according to the method of Burstall (1936). It was oxidized to the corresponding ruthenic compound by the addition of about 10 mg. of PbO_2 and 0·5 ml. of conc. HNO_3 to 10 ml. of a 10^{-3} M solution. The excess PbO_2 was removed by filtration through a fine sintered glass funnel.

Osmous tris-dipyridyl chloride ($Os(dipy)_3Cl_2$, $6H_2O$). This green compound was prepared according to Burstall, Dwyer & Gyarfas (1950). It was oxidized in solution to the red trivalent compound by HNO_3. The concentration of the solution of the trivalent compound was determined by spectrophotometric titration with $K_4Fe(CN)_6$.

Chlorine dioxide. A solution was obtained by heating a moist mixture of oxalic acid and $KClO_3$ and passing the gas into water.

Sodium hypochlorite was made by the double decomposition of $Ca(OCl)_2$ (bleaching powder) and Na_2CO_3.

All other inorganic reagents, such as KIO_4 and $NaClO_2$ were of the highest grade analytical product. The methods described by George & Irvine (1952), were used in the preparation and standardization of MetMb and the buffer solutions. Spectrophotometric measurements were made with a Unicam quartz spectrophotometer. All reactions were carried out with dissolved air present in the solutions unless otherwise stated. Except where mentioned, experiments were conducted at room temperature, 18°.

RESULTS

Sodium chlorite, potassium periodate, potassium molybdicyanide and potassium chloriridate all react with MetMb under suitable conditions giving a product with the same spectroscopic characteristics as those of the intermediate compound produced by the peroxides.

Sodium chlorite. At pH 8·6 a concentration of sodium chlorite 20 times that of the MetMb was necessary to obtain 80% yield of the intermediate

compound. Under these conditions the formation was rapid, but it was also followed by fairly rapid destruction of the haemoprotein. Thus when sodium azide was added to the reaction product after reduction with potassium ferrocyanide, very little azide complex was obtained. At pH 10·0 a higher yield was obtained and the rate of haemoprotein destruction was slower.

Potassium periodate. The reaction at pH 8·6 was extremely slow, requiring about 30 min. for completion even with a 200-fold excess of potassium periodate. Fig. 1 shows the spectrum of the product. Comparison with the spectrum of the intermediate compound obtained from hydrogen peroxide and MetMb at this pH suggests that 80–85 % is formed in the periodate reaction. Reduction with potassium ferrocyanide followed by the addition of sodium azide showed that 80–85 % of the original MetMb was recoverable, indicating a destruction of 15–20 % of the MetMb. The extent of destruction was greater in more acid solutions. Thus the spectra of the CO-haemochromogen obtained from the products of the periodate reaction at pH's 8·6 and 8·2 showed that 85 % of the original MetMb was recovered at pH 8·6 but only about 60 % at pH 8·2. At pH 6·0 no CO-haemochromogen was obtained.

Potassium persulphate. The addition of potassium persulphate to MetMb, even in large excess, caused no perceptible change in its spectrum. If, however, after the addition of potassium persulphate, silver nitrate solution was added to give a 10^{-5} M solution, there was a rapid colour change from brown to reddish violet indicating the formation of the intermediate compound. This was followed by destruction of the MetMb and finally the formation of a precipitate, presumably of silver hydroxide. Because of this it was not possible to confirm spectrophotometrically the formation of the intermediate compound with this oxidizing agent.

Potassium molybdicyanide. Oxidation of MetMb occurred only in solution with the pH above 11·0. The spectrum of the compound obtained is shown in Fig. 2. At pH 10·2, and below, the addition of potassium molybdocyanide to the intermediate compound caused complete reduction. At pH 8·6 an equimolar concentration of molybdocyanide was required but at higher pH values an excess of molybdocyanide was necessary. The spectrum of the azide complex from the reduction product at pH 8·6 is given in Fig. 3. It agrees very well with that of the azide complex from fresh MetMb.

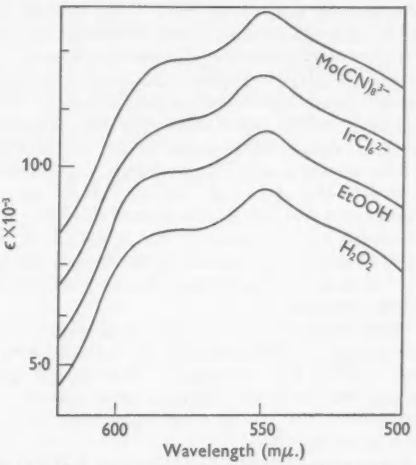

Fig. 2. Visible spectra of the compounds obtained in the reactions of metmyoglobin with hydrogen peroxide at pH 8·6, ethyl hydroperoxide (EtOOH) at pH 8·6, potassium chloriridate at pH 8·6 and potassium molybdicyanide at pH 11·1. The ordinate scale refers to the spectrum of the compound formed by hydrogen peroxide, and it has been displaced upwards by 1·25 units in each case for the spectra of the compounds formed by the other oxidizing agents.

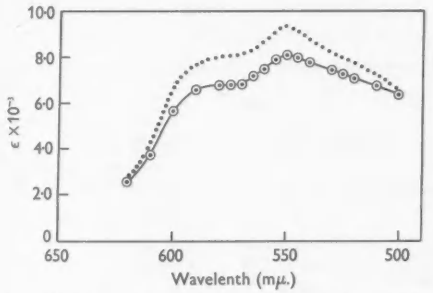

Fig. 1. Visible spectrum of the compound formed when MetMb reacts with potassium periodate at pH 8·6. The dotted curve is that of the intermediate compound formed with hydrogen peroxide, shown for comparison.

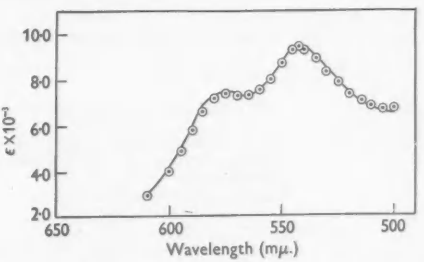

Fig. 3. Spectra of metmyoglobin–azide complex (650–500 mμ.). Smooth curve is that from stock MetMb; ⊙, that from the product of the reduction of the intermediate compound by potassium molybdocyanide at pH 8·6.

If after forming the intermediate at pH 11·1 with potassium molybdicyanide, the pH is then changed by the addition of dilute hydrochloric acid to about pH 6·0, the spectrum rapidly reverts to that of acidic MetMb. This reduction was found to be slower the more alkaline the final pH of solution.

Potassium chloriridate. Experiments at pH's 8·6 and 10·0 show that potassium chloriridate reacts very rapidly with MetMb, whether air is present or not, to give an intermediate compound whose absorption spectrum is identical with that obtained, under optimum conditions, using hydrogen peroxide or alkyl hydroperoxides (Fig. 2). Because of the instability of the intermediate compound formed under these conditions, it was necessary to make only a few optical density measurements on each of a series of solutions prepared identically. Complete regeneration of the MetMb occurred in about 3 hr., compared with about 48 hr. in the hydrogen peroxide system and about 12 hr. in the alkyl hydroperoxide system. This instability appeared to be due to the action of reducing substances produced by hydrolysis of potassium chloriridite. This explanation is supported by the slow reduction of the intermediate compound by potassium chloriridite, even at pH 8·6 where potassium chloriridate oxidizes MetMb.

With 5×10^{-6} M concentrations of both MetMb and potassium chloriridate at 18°, at least 97 % of the reaction had occurred within 20 sec. after mixing. The velocity constant must therefore be greater than $3{\cdot}2 \times 10^5$, or to the nearest power of ten, 10^5 l.mole^{-1}sec.$^{-1}$.

Spectrophotometric titrations of MetMb with potassium chloriridate at pH 8·6 were carried out by adding various amounts of stock chloriridate solution to 5×10^{-5} M MetMb solution and estimating the amount of Fe_p^{IV} from optical density measurements at 549 mμ. Fig. 4 shows that there is a linear relationship between the amount of Fe_p^{IV} formed and the chloriridate added; in this particular case a mole ratio (r) of chloriridate to metmyoglobin of 1·37 was required for complete formation of the intermediate.

Since the intermediate compound is a single equivalent oxidation product of the MetMb a ratio of 1·0 would be expected, and values of r greater than 1·0 indicate that the reaction is accompanied by side reactions involving chloriridate. This could be either hydrolysis to an oxidant not powerful enough to oxidize the MetMb or reduction of the potassium chloriridate by other reducing groups, either on the protein or present as impurities in the preparation. Titrations carried out with potassium chloriridate made up in distilled water or in dilute hydrochloric acid, with and without addition of potassium chloride, were therefore compared; any hydrolysis should be suppressed in acid solution or by chloride ion, and lower values for r should be obtained. The results (Table 1) show that r varies only from 1·30 to 1·62, and the values for the solutions in which hydrolysis should have been suppressed, had it been an important factor, are only just significantly lower than the others. This test would not reveal a rapid hydrolysis occurring in the

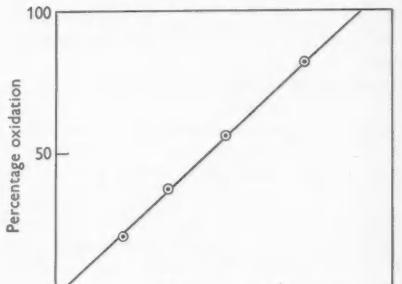

Fig. 4. Plot of percentage oxidation against the ratio [$IrCl_6^{2-}$]/[MetMb] for the reaction at pH 8·6. MetMb concentration is $4{\cdot}73 \times 10^{-5}$ M.

Table 1. *Titration of metmyoglobin with potassium chloriridate at* pH 8·6

Conditions	Ratio: K_2IrCl_6 consumed / Intermediate compound formed
Aqueous solution of K_2IrCl_6	1·62
Aqueous solution of K_2IrCl_6: fresh solution for each titration	1·53
K_2IrCl_6 in 2×10^{-3} M-HCl	1·30
K_2IrCl_6 in 4×10^{-3} M-HCl	1·43
K_2IrCl_6 in 5×10^{-3} M-HCl	1·46
K_2IrCl_6 in 5×10^{-3} M-HCl; 0·4 M-KCl present in titration mixture	1·48
K_2IrCl_6 in 5×10^{-3} M-HCl: MetMb titrated and allowed to stand. After spontaneous reduction of intermediate compound was complete (in about 3 hr.), the MetMb was again titrated	1·37

buffer solution used for the oxidation. However, a spectrophotometric comparison between solutions of potassium chloriridate made up in dilute hydrochloric acid and in the buffer gave no indication of any rapid reaction. The values of r greater than unity must therefore be due to reduction and not hydrolysis of the potassium chloriridate.

An attempt was made to confirm this by treating MetMb with potassium chloriridate in the molar proportion of 1·5:1 and then allowing the intermediate to decompose completely before carrying out the usual titration. The removal of reducing matter by this pre-treatment might have given lower titration values and a value of r nearer unity. The value 1·37 (Table 1) was not significantly different from those with fresh MetMb. However, this titration is complicated by the presence of a more powerful reducing agent formed by hydrolysis of the chloriridite ions remaining from the first treatment: this would tend to give a high value of r.

In more acid solutions, increasingly greater quantities of potassium chloriridate were required to give full formation of the intermediate compound; at pH 6·82 a fivefold excess yielded only about 50 %. This might be due either to the simultaneous removal of the greater part of the chloriridate ion in side reactions, or to the setting up of an equilibrium

$$\underset{(a-x)}{Fe_p^{3}(H_2O)} + \underset{(b-x)}{IrCl_6^{2-}} \rightleftharpoons \underset{(x)}{Fe_p^{IV}} + \underset{(x)}{IrCl_6^{3-}}. \qquad (2)$$

Estimates of the potassium chloriridate concentrations, after compound formation, were obtained from optical density measurements at 518 mμ., an isosbestic wavelength for MetMb and the intermediate compound, where the chloriridite ion has negligible absorption. The measurements showed that nearly all the potassium chloriridate remained unreacted, which is evidence for an equilibrium reaction.

The equilibrium constant,

$$K = [Fe_p^{IV}] \times [IrCl_6^{3-}]/[Fe_p^{3}(H_2O)] \times [IrCl_6^{2-}],$$

was determined from optical density measurements on a series of solutions that contained the same initial MetMb concentration, $4{\cdot}56 \times 10^{-5}$ M, and various potassium chloriridate concentrations. Measurements were made at two wavelengths, 518 and 580 mμ., from which the amount of unreacted potassium chloriridate and the percentage formation of the intermediate could be obtained. The results are given in Table 2. The equilibrium constant cannot be calculated solely on the basis of reaction (2), since this does not allow for the possible reduction of some of the potassium chloriridate in side reactions, already observed at pH 8·6. From the data given in the Table it follows that for the initial concentrations of potassium chloriridate of 6·64, 9·96, 13·28 and $24{\cdot}90 \times 10^{-5}$ M, 1·43, 1·52, 1·56 and $2{\cdot}13 \times 10^{-5}$ M respectively was reduced in this manner. Denoting this concentration by y, reference to reaction (2) shows that $[IrCl_6^{2-}]$ and $[IrCl_6^{3-}]$ at equilibrium are now given by $(b-x-y)$ and $(x+y)$. On this basis good agreement is obtained for the equilibrium constant

$$K = x(x+y)/(a-x)\,(b-x-y),$$

shown in the last column of Table 2, the mean value being $0{\cdot}20 \pm 0{\cdot}01$.

The side reaction appears to be limited in extent and little affected by changing hydrogen-ion concentration. At pH 8·6 the results in Table 1 show that between 0·30 and 0·62 mole of potassium chloriridate is reduced/mole of MetMb oxidized to the intermediate compound. At pH 6·82 the above values for y give mole proportions of 0·31, 0·33, 0·34 and 0·47. Since this mole proportion is less than unity, and since the fraction is hardly altered as the concentration ratio $[IrCl_6^{2-}]/[MetMb]$ is increased, it is very likely that reducing matter present in the MetMb preparation is responsible rather than oxidizable groups on the MetMb itself. Even if there were only one such group, a mole proportion of at least 1·0 and probably 2·0 should be obtained since the chloriridate ion is a single equivalent oxidizing agent.

Other oxidizing agents. Chlorine dioxide and sodium hypochlorite did not give the intermediate compound, but caused considerable destruction of the MetMb as shown by the small amount of azide complex obtained after reduction by potassium

Table 2. *Determination of the equilibrium constant* K *for the metmyoglobin–potassium chloriridate reaction*

Phosphate buffer solution, pH 6·82 and $I = 0{\cdot}042$, 20·4°. Optical density measurements at 518 and 580 mμ. All concentrations are in units 10^{-5} M; initial MetMb concentration, $a = 4{\cdot}56$.

Initial $IrCl_6^{-2}$ concn. (b)	% Fe_p^{IV} formed	Concn. of Fe_p^{IV} (x)	Concn. of $IrCl_6^{2-}$ at equilibrium $(b-x-y)$	Equilibrium constant (K) $\dfrac{x(x+y)}{(a-x)\,(b-x-y)}$
6·64	23·9	1·09	4·12	0·19
9·96	31·0	1·41	7·03	0·19
13·28	37·6	1·72	10·00	0·20
24·90	49·8	2·27	20·5	0·21

Mean $0{\cdot}20 \pm 0{\cdot}01$.

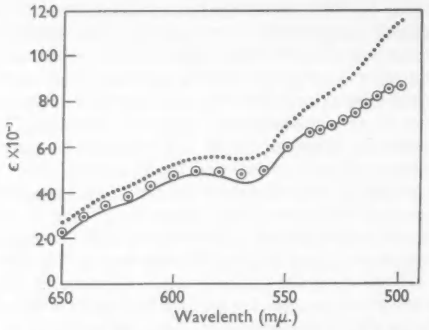

Fig. 5. Spectra obtained with the metmyoglobin–ruthenic tris-dipyridyl complex system. The dotted curve is the visible spectrum of the product of the reaction of MetMb with $Ru(dipy)_3Cl_3$ at pH 8·6; the smooth curve is that of MetMb alone at pH 8·6, ⊙ represents the curve obtained when the contribution of the absorption of $Ru(dipy)_3Cl_2$, i.e. the ruthenous complex, is subtracted from the first curve.

ferrocyanide and addition of sodium azide. The ruthenic and osmic tris-dipyridyl complexes did not give the intermediate at any pH, although having oxidation–reduction potentials of 1·30 and 0·87 v (Dwyer, 1949; Dwyer, Gibson & Gyarfas, 1950) it was to be expected that they would, since potassium chloriridate and molybdicyanide are effective with oxidation–reduction potentials of 1·0 and 0·7 v respectively. However, the ruthenic complex, and presumably the osmic complex too, is reduced in the system, for the resultant spectrum corresponds to a mixture of MetMb and the ruthenous complex, as shown in Fig. 5. This behaviour suggests that other reducing groups in the system react with cationic oxidizing agents in preference to the haematin prosthetic group of metmyoglobin, in contrast to the anionic oxidizing agents which react preferentially to give the intermediate compound. Indirect evidence which supports this explanation was obtained in a series of experiments that showed that the amino acids tryptophan, tyrosine and cysteine reduce the ruthenic and osmic complexes rapidly, whereas the chloriridate ion is reduced much more slowly.

Although the osmic complex failed to oxidize MetMb, the osmous complex reduced the intermediate at all pH values below about 8·6.

DISCUSSION

Our observations show that the single equivalent oxidation of metmyoglobin occurs with oxidizing agents other than peroxides. This could be due to the direct action of the oxidizing agent; or it could still be attributed to the action of a peroxide, formed, for instance, as a consequence of the attack of the oxidizing agent on a reducing group of the protein. Such an attack could give a radical as the primary product which, in the presence of molecular oxygen, could react to yield a peroxide. The latter mechanism is very unlikely because normal precautions to remove oxygen did not affect the production of the intermediate compound, and it is ruled out entirely in the case of potassium chloriridate and molybdicyanide by the speed of their reactions. Hydrogen peroxide and ethyl hydroperoxide react with acidic MetMb with velocity constants of about $1{\cdot}7 \times 10^2$ and $1{\cdot}7 \times 10^3$ l. mole^{-1} sec.$^{-1}$ at 18° respectively (George & Irvine, 1953*b*), whereas the electron transfer reagents react with velocity constants greater than 10^5 l. mole^{-1} sec.$^{-1}$, even with pH between 9 and 11. Under these conditions, where the MetMb is predominantly in its alkaline form, peroxides react more slowly than they do with acidic MetMb. Although peroxide formation might play a part in the action of some oxidizing agents, there is no doubt that the intermediate compound can be formed by direct oxidation, and that the special structural features of peroxides are not essential.

The very rapid formation with the electron transfer oxidizing agents, potassium chloriridate and molybdicyanide, strongly suggests that the atoms required for the structure of the intermediate compound are already present in metmyoglobin. The simplest interpretation is a reaction involving either electron or hydrogen atom removal, e.g.

$$M \rightarrow M^+ + e^-, \quad (3)$$

or

$$MH \rightarrow (M+H) \rightarrow M. + H^+ + e^-, \quad (4)$$

where M or MH represent the metmyoglobin and M^+ or M. the intermediate compound. The electron is then taken up by the $IrCl_6^{2-}$ or $Mo(CN)_8^{3-}$ ion. The observation that these oxidizing agents have certain threshold pH values, below which the intermediate compound is not formed, provides a means of obtaining a rough value of the effective oxidation–reduction potential of the Fe_p^{IV}/Fe_p^{3+} couple, i.e. reaction (3) or (4). The oxidation–reduction potential of the chloriridate couple is 1·017 v according to Dwyer, McKenzie & Nyholm (1944), and that of the molybdicyanide couple is 0·74 v (Kolthoff & Tomsicek, 1936), a value which we confirmed for a pH of 11·0 by potentiometric titration. These potentials are independent of pH, since the reactions simply involve the transfer of an electron. Since potassium molybdocyanide reduces the intermediate compound at pH 10·2 and molybdicyanide forms it from MetMb at pH values greater than 11·0, the effective potential (E_0') of the couple Fe_p^{IV}/Fe_p^{3+} may be fixed at about 0·7 v at pH 11·0. Its value at pH 6·82 may be calculated

from the equilibrium constant of the MetMb–K_2IrCl_6 reaction and the redox potential of the chloriridate couple using the equation

$$E_0' = 1{\cdot}017 - \frac{RT}{nF} \ln K, \qquad (5)$$

putting $n = 1$. This gives a value of E_0' at pH 6·82, 20·4° and ionic strength (I) = 0·042, of 1·06 v. Thus the effective oxidation–reduction potential of the Fe_p^{IV}/Fe^{3+} couple is pH dependent, which would be the case if one or more hydrogen ions were formed along with the intermediate compound, as in the generalized reaction (4) above.

Fig. 6 shows the plot of E_0' against pH. Two lines are shown in the figure, both having the point at pH 6·82 in common. One is for a single hydrogen-ion dependence and the other is for two hydrogen ions changing to a single hydrogen-ion dependence at pH 9·0, the pK of MetMb (Theorell & Ehrenberg, 1951; George & Hanania, 1952). The experimental values rule out consideration of any other type of dependence, and in fact it can be seen from Fig. 6 that even the single hydrogen dependence is not possible. The fact that the experimental data fit a variation of E_0' with pH which changes its slope at the pK of MetMb is in accord with the general

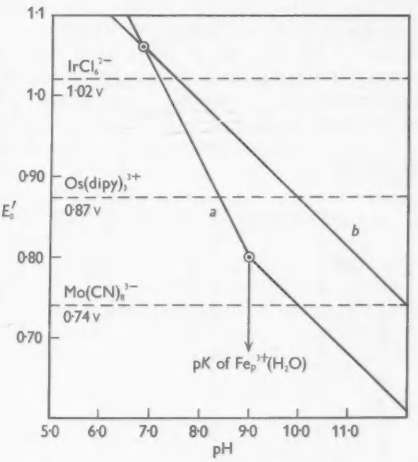

Fig. 6. Two variations of E_0' (v) with pH for the couple Fe_p^{IV}/Fe_p^{3+}, which are obtained on the basis of the equilibrium constant of the MetMb–K_2IrCl_6 reaction at pH 6·82, and the experimental observations that (i) $Os(dipy)_3^{2+}$ reduces Fe_p^{IV} at all pH values below about pH 8·6, (ii) $Mo(CN)_8^{4-}$ reduces it below pH 10·2, and (iii) $Mo(CN)_8^{3-}$ oxidizes MetMb above pH 11·0. (*a*) A line with a change in slope from $-2 \times 0{\cdot}06$ to $-1 \times 0{\cdot}06$ at the pK of acidic MetMb. (*b*) A line with a slope of $-1 \times 0{\cdot}06$ throughout.

chemical behaviour of reversible electrode systems where ionization occurs, e.g. the quinol–quinone system. It must be borne in mind, however, that this is merely the simplest interpretation of the data, for no consideration has been taken of haem-linked ionizing groups which if present could alter the slope of the E_0' versus pH curve over a range of about half a pH unit on either side of their pK values.

Finally it is of interest to see what light these experimental results throw on the structure of the intermediate compound. George (1952) suggested four possible structures. Of these, however, we may rule out the simple ion of quadrivalent iron, Fe_p^{4+}, as well as the structure in which an electron is removed from the π-orbital of the conjugated ring system, since these oxidation reactions would not involve hydrogen ions. There are left two possibilities, derivatives of quadrivalent iron and radical structures. Measurements of the number of hydrogen ions produced when the intermediate compound is formed and consumed when it is reduced back to MetMb, favour the ferryl ion structure, Fe_pO^{2+} (George & Irvine, 1954); these experiments will be described in forthcoming papers.

The work of Polonovski, Jayle, Glotz and Fraudet

These authors were the first to consider in detail how a mechanism involving a higher oxidation state could explain certain haemoprotein reactions, just as in the earlier theories of Manchot and Job (see, for example, Hale, 1929) the formation of tetravalent and pentavalent iron compounds was suggested to account for the reactions of ferrous and ferric salts with peroxides and oxygen.

They discussed the formation and reactions of the methaemoglobin intermediate on the basis that it was a tetravalent iron derivative, and drew attention to the possibility that peroxidase action might also involve such a compound (Polonovski, Jayle & Glotz, 1938, 1939; Jayle, 1939; Polonovski & Jayle, 1939; Polonovski, Jayle & Fraudet, 1941*a*, *b*).

Although agreeing on the fundamental point that the intermediate compound is a higher oxidation state of MetHb (or MetMb), we have put forward a different explanation for the reaction with peroxides. Polonovski *et al.* (1939) confirmed that approximately 1·5 moles of peroxide are consumed in forming one mole of the intermediate compound per methaemoglobin (MetHb) iron atom, as had been reported by Keilin & Hartree (1935), and they proposed the following mechanism to account for it:

$$\underset{\text{(MetHb)}}{\text{GlFe}^{3+}\!\!<\!\!\begin{matrix}\text{O}\\ \|\\ \text{O}\end{matrix}} + 1{\cdot}5\ \text{RO}_2\text{H} \rightarrow \underset{\text{(A)}}{\text{GlFe}^{4+}\!\!<\!\!\begin{matrix}\text{O—R}\\ \\ \text{O—H}\end{matrix}} + 1/2\ \text{ROH} \qquad (6)$$

For the corresponding reaction with oxyhaemoglobin (HbO_2) they suggested,

$$GlFe^{2+}\langle \overset{O}{\underset{O}{\|}} + RO_2H \rightarrow GlFe^{4+}\langle \overset{O}{\underset{O}{\|}} + ROH, \qquad (7)$$

(HbO_2) (B)

which accounted for their observation that there was a maximal colour change when the reactants were mixed in a 1:1 mole ratio. Both (A) and (B) denote the intermediate compound in these equations. Noting that the products from MetHb and HbO_2 had slightly different spectra, although the colours were similar, they proposed that (A) and (B) took part in an equilibrium reaction in which oxygen replaced the peroxide molecule bound to the tetravalent iron.

$$GlFe^{4+}\langle \overset{O—R}{\underset{O—H}{}} + O_2 \rightleftharpoons GlFe^{4+}\langle \overset{O}{\underset{O}{\|}} + RO_2H. \qquad (8)$$

(A) (B)

To explain the consumption of peroxide in excess of the one equivalent (half mole) required for oxidation, we suggested instead that a transient oxidizing entity X was first formed

$$Fe_p^{3+}(H_2O) + H_2O_2 \rightarrow Fe_p^{IV} + X, \qquad (9)$$

and, depending on the extent to which it then reacted with endogenous reducing matter or with peroxide, a consumption ratio of unity, or greater, would then follow (George & Irvine, 1952, 1953*a*). In two respects this mechanism is more acceptable. Unlike reaction (6) it accounts for the high consumption ratios, greater than three, obtained with alkyl hydroperoxides in alkaline solution, and also reaction (9) is a simple bimolecular reaction. Reaction (6), on the other hand, expresses an overall reaction at a specified consumption ratio of 1·5 and is not a reaction capable of correlation with kinetic measurements since fractions of a mole participate.

A potentiometric titration was used to determine the number of equivalents involved per iron atom when the intermediate is formed from MetHb (Polonovski *et al.* 1941*a*). The results are given in Fig. 7: 10^{-3} M MetHb was rapidly titrated with ethyl hydroperoxide in phosphate buffer of pH 6·5 at 1° to avoid as far as possible the rapid spontaneous decomposition of the intermediate compound that was very apparent at room temperature. The potential rose from 185 mv reaching a limiting value of 385 mv when 1·25 equivalents, i.e. 0·63 mole, of ethyl hydroperoxide had been added per mole of MetHb, as shown in curve *a*. On the addition of ascorbic acid the potential dropped and about 1·0 equivalent, i.e. 0·5 mole, per mole MetHb was needed for the potential to return to about its original value, as shown in curve *b*. Further addition of ascorbic acid only produced haemoglobin. At first sight these titrations appear to demonstrate the one equivalent oxidation of MetHb to a higher oxidation state: but there are serious quantitative discrepancies. First, there is a difference between the potential values in curves *a* and *b* amounting to as much as 100 mv in the early stages of the reduction. This large difference casts doubt on the validity of the measurements, and if it arose through decomposition of some of the intermediate compound, then the results are not quantitative. Secondly, the end point of the titration suggests that 1 mole of the intermediate compound is produced by reaction with 1·25 equivalents, i.e. 0·63 mole, of peroxide, which is at variance with Keilin & Hartree's results, and their observations, which showed that more than 1 mole of peroxide is needed. It is unlikely, therefore, that under the conditions of the potentiometric titration full formation of the intermediate compound was obtained. For these reasons we do not believe that the titrations in Fig. 7 constitute a satisfactory proof of the single equivalent oxidation, although it might well be possible to find conditions where the method could be used.

In solutions of pH 6·5 it has recently been shown that additional reactions occur giving products that cannot be reduced to the original MetHb, or MetMb (Dalziel & O'Brien, 1952; George & Irvine, 1952). Whilst this further complicates the interpretation of the potentiometric titration, it offers a more likely explanation for the differences noted between the reaction of MetHb and of HbO_2 than the equilibrium reaction (8), in which an oxygen molecule is co-ordinated to the tetravalent iron atom. There was no direct evidence for the bonding of oxygen in this way, or to methaemoglobin as specified in reaction (6).

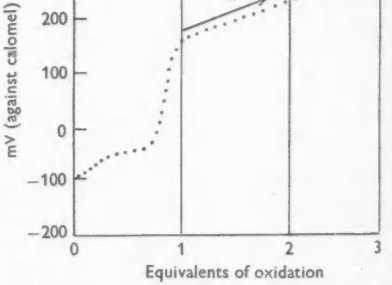

Fig. 7. Potentiometric titration of methaemoglobin with ethyl hydroperoxide (taken from Polonovski *et al.* 1941*a*). *a*, MetMb with ethyl hydroperoxide; *b*, reduction of the compound formed with ascorbic acid.

SUMMARY

1. Sodium chlorite, potassium periodate, potassium persulphate with silver ions present, potassium molybdicyanide and potassium chloriridate all react with metmyoglobin to give compounds with the same spectroscopic characteristics as those of the intermediate compound produced by the peroxides, a one-equivalent oxidation product which can be denoted by the symbol Fe_p^{IV}.

2. The oxidizing agents containing oxygen, e.g. potassium periodate, react slowly with metmyoglobin, but the reactions with the electron transfer reagents are extremely rapid with velocity constants greater than 10^5 l. mole^{-1}sec.$^{-1}$, even in alkaline solutions.

3. Potassium molybdicyanide only oxidizes metmyoglobin above pH 11·0. At pH 8·6 and below, an equimolar concentration of potassium molybdocyanide reduces the intermediate compound.

4. In the reaction between potassium chloriridate and metmyoglobin at pH 8·6 the plot of percentage oxidation against the ratio $[IrCl_6^{2-}]/[MetMb]$ is linear, a mole ratio of about 1·5 being required for complete reaction.

5. In solutions of pH about 7·0 the intermediate compound is formed in an equilibrium reaction with potassium chloriridate, the constant (K) having a value of $0{\cdot}20 \pm 0{\cdot}01$ at pH 6·82, 20·4° and $I = 0{\cdot}042$.

6. The cationic electron transfer oxidizing agents, ruthenic and osmic tris-dipyridyl complexes, do not form the intermediate compound with metmyoglobin. They appear to react preferentially with other groups rather than with the iron atom. However, the corresponding osmous complex reduces the intermediate compound at all pH values below about 8·6.

7. The variation of E_0' with pH for the Fe_p^{IV}/Fe_p^{3+} couple, inferred from the threshold pH values observed in the action of several of the oxidizing and reducing agents, is discussed together with its bearing on possible structures for the higher oxidation state.

8. The papers of Polonovski, Jayle, Glotz & Fraudet, who suggested between 1938 and 1941 that quadrivalent iron compounds participate in haemoprotein reactions, are critically examined.

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Aerobic Metabolism of the Muscle of *Locusta migratoria*

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During investigations of the aerobic metabolism of some invertebrates (Rees, 1952, 1953) the muscle of *Locusta migratoria* was examined. Barron & Tahmisian (1948) have shown that whole muscle fibres of the cockroach, *Periplaneta americana*, contain a glycolytic system and enzymes for the oxidation of citric acid cycle intermediates. Breis and acetone powders of *Drosophila* have also been found to contain enzymes for the oxidation of a number of citric acid cycle intermediates (Spirtes, 1951).

Watanabe & Williams (1951) described the isolation of sarcosomes from flight muscles of *Drosophila funebris* and of the blowfly, *Phormia regina*, and showed them to possess high respiratory activity. They concluded that these particles resembled mammalian mitochondria.

Sacktor (1953) was unable to demonstrate oxidative phosphorylation with insect sarcosome preparations. However, Lewis & Slater (1953) reported oxidative phosphorylation to occur with sarcosome preparations of the blowfly *Calliphora erythrocephala*.

In this present investigation, it has been shown that sarcosomes from *Locusta migratoria* possess citric acid cycle activity and the capacity for oxidative phosphorylation. The conditions necessary for such activity have been investigated, and it has been demonstrated that the addition of ethylenediaminetetraacetic acid is necessary for oxidative phosphorylation and that oxidation of citric acid is very slow unless either triphosphopyridine nucleotide or a mixture of diphosphopyridine nucleotide and adenosine triphosphate are added.

EXPERIMENTAL

All the experiments reported in this paper were carried out on adult male or female *L. migratoria*. The animals were kept at 32°.

Enzyme preparations

Cell-free tissue suspensions. By means of scissors, the head, abdomen and appendages were removed from the thoraces of the insects. The thorax was opened along the mid-dorsal line and the gut removed. The thoraces and appendages, without further dissection, were chopped with scissors in ice-cold 0·2 M sucrose solution and then ground in an ice-cold Potter all-glass grinder. The suspension was diluted with 0·2 M sucrose to give a final concentration of approximately 10% tissue and then centrifuged at 500 *g* for 5 min. to remove the chitin, cellular debris and nuclei.

For studying the synthesis of citric acid, KCl suspensions were prepared by the above method substituting 0·5% (w/v) KCl for 0·2 M sucrose.

Sarcosome suspensions. A muscle suspension prepared as described above was centrifuged at 10000 *g* for 10 min. at 2° in a Servall SS-1-a high-speed centrifuge. The reddish brown mass of sarcosomes was washed by suspending in 0·2 M sucrose and again centrifuging. The sarcosomes from eight adult locusts were finally suspended in 6–7 ml. of 0·2 M sucrose.

Microscopic examination of such preparations showed a practically pure preparation of sarcosomes, 3–8 μ. in diameter. The first supernatant from the high-speed centrifuging was found to be almost free of sarcosomes.

Myokinase was prepared by the method of Colowick & Kalckar (1943).

Hexokinase was obtained from yeast by the method of Berger, Slein, Colowick & Cori (1946), as described by Cross, Covo, Taggart & Green (1949). The preparation was stored as a frozen solution at −10°. The conditions for autolysis of baker's yeast were those described by Bailey & Webb (1948).

Analytical methods

Inorganic orthophosphate was determined by the method of Fiske & Subbarow (1925) and α-oxoglutarate and pyruvate by the method of Friedemann & Haugen (1943). Citrate was determined by the method of Weil-Malherbe & Bone (1949). Succinate was determined by the method of Krebs (1937). Oxygen uptakes were determined manometrically; the gas phase was air.

Materials

Triphosphopyridine nucleotide (TPN) was isolated by the method of LePage & Mueller (1949).

Flavin-adenine dinucleotide (*FAD*) was prepared by a modification of the method of Warburg & Christian (1938).

Yeast juice. Fresh baker's yeast was washed and centrifuged 3 times with 4 vol. of 1% NaCl. The washed yeast was added to an equal volume of water maintained at 90°. After the final addition of yeast, the temperature was maintained for 2 min. at 90°. The yeast suspension was cooled rapidly and centrifuged and the clear, bright yellow supernatant was stored at −10°.

Coenzyme A (*CoA*), *thiamine pyrophosphate* (*TPP*) *and glutathione* (*GSH*) were commercial products. Other cofactors and reagents were gifts.

Standard medium. Final concentrations in the Warburg flasks were: KCl (0·025 M), Mg^{2+} (0·0066 M), adenosine 5′-phosphate (0·001 M), cytochrome *c* (10^{-5} M), sodium phosphate buffer, pH 7·4 (0·014 M), and substrate as indicated in the tables. Final volume, 3 ml. The enzyme preparation (0·5 ml.) was added in 0·2 M sucrose solution. 0·1 ml. 20% KOH was placed in the centre well with filter paper. Temperature equilibration was carried out for 5 min.

RESULTS

Reactions of citric acid cycle

The effect of the addition of various glycolytic and citric acid cycle intermediates on the respiration of muscle suspensions in 0·2 M sucrose was investigated (Table 1). All the substrates tested were found to increase the rate of respiration. The endogenous respiration of the suspension varied greatly from preparation to preparation. Dialysis of muscle suspensions against 0·2 M sucrose resulted in a diminution of the endogenous respiration and of the ability of the preparation to oxidize citric acid cycle intermediates.

Table 1. *The effect of glycolytic and citric acid cycle intermediates on the respiration of muscle suspensions in* 0·2 M *sucrose*

System: standard medium containing 30 μmoles of substrate (100 μmoles in case of succinate). Tissue suspension added equivalent to 1 mg. N. Duration 30 min.; 32° in air.

Expt.	Substrate	O_2 uptake (μl.)
1	Nil	20
	Fructose 6-phosphate	49
	Hexose diphosphate	47
	Phosphoglyceric acid	39
	L-Malate	54
	Succinate	160
2	Nil	51
	Oxaloacetate	74
	α-Oxoglutarate	105
	Fumarate	80
	cis-Aconitate	74
	Pyruvate	59

Table 2. *The effect of malonate and arsenite on the respiration of muscle suspensions in* 0·2 M *sucrose*

System: standard medium with final concentrations of 0·033 M malonate or 0·007 M arsenite. Other additions as indicated. 60 min. respiration at 32° in air. Tissue suspension added equivalent to 1 mg. N.

Additions	O_2 uptake (μl.)	Keto acid accumulation
Nil	150	—
Malonate	7	—
Malonate + 5 μmoles L-malate	15	—
Malonate + 10 μmoles L-malate	33	α-Oxoglutarate (1·2 μmoles)
L-Malate (25 μmoles)	190	—
Arsenite	Nil	Pyruvate (0·5 μmole)
Arsenite 2 μmoles L-malate	17	Pyruvate (2·0 μmoles)
Arsenite 5 μmoles L-malate	28	Pyruvate (3·0 μmoles)

Table 3. *Synthesis of citrate by muscle suspensions*

System: 0·0066 M-Mg^{2+}, 0·014 M sodium phosphate buffer (pH 7·4), 0·0015 M-ATP, 20 μmoles each of oxaloacetate and pyruvate. Reactions carried out anaerobically in Thunberg tubes. Tissue added as 1 ml. of muscle suspension in 0·5% KCl (≡1 mg. N). Duration of experiment, 30 min.

Substrate	Citrate synthesis (μmoles) Expt. 1	Expt. 2
Nil	0	0·2
Pyruvate + oxaloacetate	2·0	3·1

Table 4. *The accumulation of succinate by muscle suspensions*

System: standard medium containing 0·033 M malonate. 50 min. incubation at 32°. Tissue suspension in 0·2 M sucrose equivalent to 1 mg. N. 10 μmoles α-oxoglutarate used.

Additions	O_2 uptake (μatoms)	α-Oxoglutarate utilized (μmoles)	Succinate formed (μmoles)
Nil	1·0	—	—
α-Oxoglutarate	3·7	3·0	3·0
α-Oxoglutarate	4·1	3·8	3·4

The effects of malonate (0·033 M) and arsenite (0·007 M) on muscle suspensions were tested both in the presence and absence of added dicarboxylic acids (Table 2). The inhibition of the endogenous respiration by malonate was diminished by the addition of L-malate and α-oxoglutarate was found to have accumulated. The addition of arsenite resulted in complete inhibition of the endogenous respiration but under these conditions the system was capable of oxidizing L-malate to pyruvate, which accumulated.

Table 3 shows the synthesis of citrate from oxaloacetate and pyruvate. The reaction was investigated in Thunberg tubes under anaerobic conditions to prevent oxidation of the citrate formed.

The reaction α-oxoglutarate → succinate was studied in the presence of malonate to prevent further oxidation of succinate. Table 4 shows the results of two experiments.

These experiments indicate that the muscle of *L. migratoria* contains enzymes which will oxidize citric acid cycle intermediates. The inhibition of the endogenous respiration in the presence of malonate and arsenite further suggests that the citric acid cycle plays a part in the endogenous respiration.

The oxidation of citric acid cycle intermediates by sarcosomes from whole muscle suspensions was investigated. The rates of oxidation are l[illegible] (Table 5).

The addition of ATP, ADP, FAD, GSH, TPP or CoA, singly or in combination, had no effect on the rates of oxidation. DPN was found however to increase the rate of oxidation (Table 5).

Table 5. *The effect of various cofactors and protein-containing solutions on the respiration of sarcosomes*

System: standard medium, with further additions as shown. CoA (5 Lipmann units), ADP and DPN (3 μmoles) and TPP (100 μg.) when added. 30 min. incubation at 32° in air. 0·5 ml. of supernatant or yeast juice added. Sarcosomes added equivalent to 0·3 mg. N and muscle suspension equivalent to 1·5 mg. N (0·3 mg. of sarcosome N derived from 1·5 mg. of cell-free suspension N). Succinate (100 μmoles) and L-glutamate, α-oxoglutamate or L-malate (30 μmoles), where added.

Expt.	Substrate	Enzyme preparation	Additions	O_2 uptake (μatoms)
1	Succinate	Cell-free muscle suspension	Nil	6·5
		Sarcosomes	Nil	14·7
		Sarcosomes	Supernatant	20·2
		Sarcosomes	ADP	15·3
		Sarcosomes	CoA + TPP	15·3
2	L-Glutamate	Cell-free muscle suspension	Nil	13·3
		Sarcosomes	Nil	1·7
		Sarcosomes	Supernatant	7·0
	L-Malate	Sarcosomes	Nil	3·8
		Sarcosomes	Supernatant	14·0
		Sarcosomes	Boiled supernatant	6·1
		Sarcosomes	Yeast juice	9·8
		Sarcosomes	DPN	5·6
		Sarcosomes	CoA + TPP	4·4
3	L-Glutamate	Cell-free muscle suspension	Nil	13·0
		Sarcosomes	Nil	3·3
		Sarcosomes	Supernatant	14·0
		Sarcosomes	Boiled supernatant	6·8
		Sarcosomes	Yeast juice	6·9
		Sarcosomes	DPN	4·7
		Sarcosomes	CoA + TPP	3·1
	α-Oxoglutarate	Sarcosomes	Nil	4·8
		Sarcosomes	Supernatant	9·5
		Sarcosomes	Boiled supernatant	7·7
		Sarcosomes	Yeast juice	8·0
		Sarcosomes	DPN	5·5
		Sarcosomes	CoA + TPP	4·4

Table 6. *The effect of various protein-containing solutions on the oxidation of α-oxoglutarate by sarcosome preparations*

System: standard medium with or without 0·01 M α-oxoglutarate; final volume, 3 ml., including 0·5 ml. sarcosome suspension (≡0·6 mg. N) with or without 0·5 ml. yeast juice. 20 min. incubation at 32° in air.

Additions	O_2 uptake (μatoms)	α-Oxoglutarate utilized (μmoles)
α-Oxoglutarate	8·5	8·0
α-Oxoglutarate + yeast juice	16·0	14·3
Yeast juice	6·5	—
Nil	1·0	—

It was found that the addition to such sarcosome preparations of some of the supernatant fluid from which they had been isolated resulted in a large increase in the respiration (Table 5). This supernatant fluid still retained some activity after being kept at 100° for 2 min. The supernatant fluid in the absence of added sarcosomes possessed no residual respiration and was found not to show any L-glutamic dehydrogenase activity as determined manometrically in the presence of DPN and with methylene blue as carrier. The supernatant fluid effect was not lost after dialysis for 12 hr. at 0° against 0·2 M sucrose. Yeast juice was observed to increase the rate of oxidation of citric acid cycle intermediates by sarcosomes. The results are also included in Table 5. The rates of oxidation were now of the same order as whole muscle suspensions despite the fact that the yeast juice gave no oxygen uptake in the absence of added substrate. This point was confirmed by following the oxidation of α-oxoglutarate in the presence of sarcosomes and yeast juice. It was found that oxidation of α-oxoglutarate was in fact accelerated by the addition of yeast juice (Table 6).

The supernatant fluid and yeast juice (which contains protein) could exert their effects by the removal of inhibitory metals. In view of the findings of Slater & Cléland (1953), the effect of ethylenediaminetetraacetic acid (EDA) (Versene)

was studied and was found to increase the rate of respiration to the same extent as the yeast juice and supernatant fluid. Histidine, however, would not substitute for EDA in these sarcosome preparations (Table 7).

Table 7. *The effect of ethylenediaminetetraacetic acid and* L-*histidine on the oxidation of* L-*malate by sarcosome preparations*

System: standard medium with 30 μmoles L-malate. Sarcosomes added equivalent to 0·5 mg. N, and incubation at 32°.

Additions	Concn. (M $\times 10^{-4}$)	O_2 uptake (μl.) 1st 60 min.	O_2 uptake (μl.) 2nd 60 min.
Nil	—	94	25
Ethylenediaminetetra-acetic acid	3·3	111	53
Ethylenediaminetetra-acetic acid	1·0	115	49
Histidine	3·3	86	19
Histidine	1·0	102	19

Table 8. *The oxidation of citrate, acetate and octanoate by sarcosome preparations*

System: standard medium. Substrates: citrate (30 μmoles), pyruvate and acetate (20 μmoles), L-malate and octanoate (3 μmoles). Other additions: ATP (3 μmoles), DPN and TPN (1 μmole); final concn. of ethylenediaminetetraacetic acid in all cases $3{\cdot}3 \times 10^{-4}$ M. Sarcosomes added equivalent to 0·5 mg. N. Incubated for 40 min.

Expt.	Substrate	Other additions	O_2 uptake (μl.)
1	Citrate	Nil	30
	Citrate	ATP + DPN	327
	Citrate	TPN	404
2	L-Malate	Nil	37
	L-Malate + pyruvate	Nil	147
	L-Malate + acetate	Nil	74
	L-Malate + octanoate	Nil	32

Table 9. *Component requirement for oxidative phosphorylation by a* 0·2 M *sucrose muscle suspension*

Complete system: standard medium, 0·03 M glucose, 0·01 M L-glutamate, 0·014 M-NaF, 0·05 ml. hexokinase solution. Final volume, 3 ml. 30 min. incubation in air at 32°; equilibration 5 min. (allowed for in results). Tissue suspension added equivalent to 2 mg. N.

System	O_2 uptake (μatoms)	Phosphate esterified (μmoles)
Complete system	9·2	19·0
No glucose	9·2	19·0
No magnesium	9·0	19·0
No NaF	14·5	29·0
No hexokinase	7·3	14·0
No AMP	8·6	19·0
No L-glutamate	3·7	1·0

Citric acid was oxidized by sarcosome preparations only in the presence of TPN or of ATP and DPN. In the latter case presumably a synthesis of TPN had occurred. The preparations oxidized added pyruvate and acetate in the presence of a primer such as L-malic acid. It was not possible to demonstrate the oxidation of octanoic acid even in the presence of added cofactors (Table 8).

Oxidative phosphorylation in muscle

The endogenous respiration of whole muscle suspensions was found to be accompanied by the esterification of inorganic phosphate as was the oxidation of citric acid cycle intermediates. The components of the oxidative phosphorylation systems were investigated (Table 9). There is a small but significant effect of added hexokinase. It is presumed that there is present in the locust preparations a large amount of endogenous hexose and a powerful hexokinase (Leaback, 1953, private communication). This contrasts with the large

Table 10. *The effect of fluoride on oxidative phosphorylation of* 0·2 M *sucrose muscle suspensions*

System: standard medium; substrates, 30 μmoles, excepting succinate (100 μmoles); 0·05 ml. hexokinase solution; 100 μmoles glucose. Final concentration of sodium fluoride, 0·014 M. Duration of experiment 20 min. with 5 min. equilibration, at 32°. Tissue suspension added equivalent to 2 mg. N.

Expt.	Substrate	NaF	O_2 uptake (μatoms)	P esterified (μmoles)	P/O ratio
1	Nil	−	7·5	13·5	1·8
	Nil	+	3·0	Nil	0
	Succinate	−	44·0	24·5	0·56
	Succinate	+	21·0	16·5	0·79
	L-Malate	−	12·0	20·0	1·66
	L-Malate	+	5·1	10·0	1·95
	cis-Aconitate	−	8·0	10·0	1·25
	cis-Aconitate	+	4·0	3·0	0·75
2	Nil	−	1·5	Nil	0
	Nil	+	1·5	Nil	0
	L-Glutamate	−	9·5	20·0	2·1
	L-Glutamate	+	6·0	14·0	2·3
	α-Oxoglutarate	−	6·5	13·5	2·0
	α-Oxoglutarate	+	4·5	12·0	2·7

Table 11. *The effect of protein-containing solutions on oxidative phosphorylation by sarcosome preparations*

System and conditions as described in Table 10. 0·5 ml. of the supernatant from which the sarcosomes were isolated or 0·5 ml. of a yeast juice extract used. Sarcosomes added equivalent to 1·0 mg. N (NaF not added).

Expt.	Substrate	Additions	O_2 uptake (μatoms)	P esterified (μmoles)
1	Nil	Nil	0·5	Nil
	Nil	Supernatant	0·5	Nil
	Succinate	Nil	10·0	Nil
	Succinate	Supernatant	22·0	16·0
	Succinate	Boiled supernatant	15·5	6·1
	Succinate	Yeast juice	25·0	17·0
	Nil	Yeast juice	6·3	9·0
2	Nil	Nil	0·5	Nil
	L-Glutamate	Nil	3·3	Nil
	L-Glutamate	Supernatant	14·0	21·0
	L-Glutamate	Boiled supernatant	6·8	5·3
	L-Glutamate	Yeast juice	6·9	7·5
	Nil	Yeast juice	3·6	0·7
	α-Oxoglutarate	Nil	4·8	Nil
	α-Oxoglutarate	Supernatant	9·5	6·7
	α-Oxoglutarate	Boiled supernatant	7·7	7·1
	α-Oxoglutarate	Yeast juice	8·0	9·3

Table 12. *The effect of ethylenediaminetetraacetic acid and 2:4-dinitrophenol on oxidative phosphorylation by sarcosome preparations*

System and conditions as described in Table 10. Ethylenediaminetetraacetic acid (EDA) added to a final concentration of 0·001 M; 0·5 ml. yeast juice and 2:4-dinitrophenol (DNP) final concn. 10^{-4} M used. Sarcosomes added equivalent to 1·0 mg. N (NaF not added).

Expt.	Substrate	Additions	O_2 uptake (μatoms)	P esterified (μmoles)
1	Nil	Nil	0·5	Nil
	α-Oxoglutarate	Nil	11·4	10·0
	α-Oxoglutarate	EDA	15·8	32·0
	α-Oxoglutarate	Yeast juice	16·5	37·0
	α-Oxoglutarate	DNP + EDA	11·0	0·2
2	Nil	Nil	0·5	Nil
	L-Glutamate	Nil	6·7	4·0
	L-Glutamate	EDA	10·0	29·0
	L-Malate	Nil	7·3	Nil
	L-Malate	EDA	16·5	38·0
	L-Malate	DNP + EDA	16·0	3·0

effects seen with rat-liver suspensions (Lardy & Wellman, 1952). The addition of sodium fluoride is found to inhibit the respiration of muscle suspensions both in the presence and absence of added citric acid cycle intermediates; there is, however, sometimes an increase in the P/O ratio, although it was found with *cis*-aconitate that the addition of fluoride gave the opposite effect. It will be seen from Table 10 that the P/O ratios obtained were of the same order as those obtained in mammalian tissue (Judah & Williams-Ashman, 1951).

The oxidative phosphorylation in sarcosomes was investigated. There was wide variation in the ability of preparations to esterify inorganic phosphate. The addition of the pyridine nucleotides, adenine nucleotides and myokinase singly or in combination failed to elicit oxidative phosphorylation.

As in the case of respiration, both supernatant fluid and yeast juice stimulated oxidative phosphorylation (Table 11) and again EDA substituted satisfactorily for the crude extracts (Table 12). Slater & Lewis (1953) observed that 2:4-dinitrophenol (DNP) stimulated the respiration and increased the overall phosphate esterification of sarcosomes from *Calliphora erythrocephala* in the presence of hexokinase and glucose. This is in contrast to observations on mammalian mitochondria (Lardy & Wellman, 1952). In *Locusta migratoria* sarcosome preparations studied in the presence of hexokinase and glucose, DNP was without effect on the respiration but lowered the efficiency of oxidative phosphorylation (Table 12).

DISCUSSION

Suspensions of locust muscle in 0·2 M sucrose show high rates of endogenous respiration. The addition of glycolytic or citric acid cycle intermediates either increases the rate of respiration or stabilizes the initial rate. The reactions α-oxoglutarate → succi-

nate and the synthesis of citric acid from pyruvate and oxaloacetate occur in muscle suspensions. The results of studies with malonate and arsenite indicate that the major part of the endogenous respiration passes through the citric acid cycle and the activity/mg. N values for citric acid cycle substrates give figures of the same order as those obtained for cell-free sucrose suspensions of rat liver.

The muscle suspensions were found to esterify inorganic phosphate and the level of esterification was increased by the addition of citric acid cycle intermediates. The suspensions contained enough endogenous hexose to accept phosphate groups from ATP. Enough hexokinase was present in the suspension and addition of yeast hexokinase only increased the esterification of inorganic phosphate by 25%.

Different sarcosome preparations showed great variation in their ability to oxidize citric acid cycle intermediates. Variation was also found to occur in oxidative phosphorylation. The rate of respiration of a preparation in the presence of a citric acid cycle intermediate proved to be no guide to its ability to phosphorylate. High rates of respiration often did not result in phosphate esterification whereas, in contrast, a low rate of respiration might well be associated with oxidative phosphorylation. It was also found that with a given sarcosome preparation there was substrate variation, e.g. with L-malate, oxidative phosphorylation might occur, whereas with the oxidation of succinate it did not. Sacktor (1953) has shown that the inability to demonstrate oxidative phosphorylation in insect preparations was not due to a rapid breakdown of ATP.

The addition of the sucrose supernatant fluid to the isolated sarcosomes was found to confer on the preparation the power of oxidative phosphorylation. Treatment of this supernatant at 100° for 2 min. followed by centrifuging caused some loss of the activity. This effect was also displayed by yeast juice. It was considered that these protein solutions were abstracting an inhibitor from the sarcosome preparations.

Calcium is a well known inhibitor of oxidative phosphorylation (Potter, 1947; Lehninger, 1949). Slater & Cleland (1953) had found large quantities of calcium present in mammalian heart preparations and used EDA to remove it. The addition of 0·001 M EDA to the locust sarcosomes gave preparations capable of oxidative phosphorylation. There are small variations in the effect of EDA. The maximum effective final concentration varied from one preparation to another, depending on whether the sarcosomal preparation was or was not capable of oxidative phosphorylation in the absence of EDA. However, in the range $3{\cdot}3 \times 10^{-4}$–10^{-3} M EDA not more than 10% inhibition of respiration was found and in the majority of cases increases of respiration and of oxidative phosphorylation from 2 to 4 times were observed.

With sarcosome preparations in the presence of EDA the oxidation of citric acid cycle intermediates and of pyruvate and acetate was found to take place but we were unable to demonstrate manometrically the oxidation of octanoate. 2:4-Dinitrophenol was found to inhibit oxidative phosphorylation, in contrast to the effect reported by Slater & Lewis (1953) with insect sarcosomes, where increased phosphate esterification occurred in the presence of DNP.

Watanabe & Williams (1953) investigated the effects of various media on the size and organization of isolated sarcosomes of *Drosophila* and *Phormia*. Protein-containing solutions were extremely effective in preserving the sarcosomes but EDA was ineffective. These authors quote unpublished results of Sacktor which show that oxidative phosphorylation occurs with isolated sarcosomes only in the presence of proteins whereas 0·001 M EDA abolished the oxidation of α-oxoglutarate. Watanabe & Williams considered this to be in good agreement with their results.

According to the results reported in this paper, EDA substitutes for the proteins. These results with EDA confirm some of the findings of Lewis & Slater (1953) with *Calliphora erythrocephala* sarcosomes who reported oxidative phosphorylation with α-oxoglutarate as substrate in the presence of EDA.

SUMMARY

1. The oxidation of a number of citric acid cycle intermediates takes place in muscle suspensions and sarcosomes of *Locusta migratoria*.

2. Oxidation of citric acid by sarcosomes has been shown to require added triphosphopyridine nucleotide.

3. Oxidative phosphorylation has also been demonstrated in muscle suspensions and sarcosomes.

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The Titration Curve of Gelatin

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Titration curves have played a considerable part in both the determination of the amino acid composition of proteins and in studying their behaviour as acids and bases (Cohn & Edsall, 1943). A serious discrepancy was known to exist for many years between the analytically determined dicarboxylic acid content of gelatin (or collagen) and the values derived from titration. The discrepancy persisted even after it became clear that some of the dicarboxylic acid residues were present, in collagen and in certain types of gelatin, as amides. Improved determinations of the dicarboxylic acids showed that there had been errors in previous analyses. There remained, however, a discrepancy (Ames, 1952) in the content of histidine, the titration curve giving too high a value. In spite of recent improvements of methods of amino acid analysis, titration remains a valuable tool for studying the peculiarities of dissociating groups, especially when these are chemically labile; for example, where ionizing groups are blocked or modified by treatment with reagents, or where carboxylic groups are present in part as amides.

The present paper describes the experimental method and its application to two types of gelatin: this has enabled four of the five most important reactive groups to be estimated, namely carboxyl, imidazole, α-amino, guanidino.

EXPERIMENTAL

Materials. Alkali-processed ox-hide gelatin (1), isoionic point pI = 4·92. The gelatin was prepared commercially from ox hide, which had been soaked for several months in a saturated solution of $Ca(OH)_2$ at about 15°. After neutralization, gelatin was extracted from the hide using warm water (*ca.* 60°).

Acid-processed pig skin gelatin (2), pI = 9·1. The gelatin was prepared commercially from pig skin by extraction at pH 3·5–4·0, with warm water. No alkaline pre-treatment was used.

De-ionization. 4–6% (w/v) solutions of each of the two gelatins were de-ionized by passage through a mixed bed of Amberlite resins IR–120 and IRA–400 (Rohm and Haas Co., Philadelphia 5, Pa.) as described by Janus, Kenchington & Ward (1951). The pH of the solution leaving the resin bed gave the pI of the gelatin. The de-ionized solutions were set, dried and ground to give a coarse powder. The pH of the solution of the acid-processed gelatin was brought, after de-ionization, from 9·1 to *ca.* 5·0 by the addition of HCl, before drying and grinding. The ash content of the gelatins was reduced to less than 0·1% (w/w) from 2·5% for gelatin (1) and 0·5% for gelatin (2).

Preparation of solutions for titration. A weighed quantity of the de-ionized gelatin, the water content of which had been determined by drying for 18 hr. at 105° in the form of a thin film, was allowed to swell and then dissolved by gentle warming to 40°. Concentrations of 4·5% (w/v) were used. The concentration was checked by drying a weighed quantity of solution at 105° for 18 hr.

pH *standardization.* All pH measurements were made with Doran 'Alkacid' glass electrodes (L.S.B. Components Ltd., Stroud, Glos.) with a modified Cambridge pH meter (Kenchington & Ward, 1954), which enabled several electrodes to be used in succession, without re-standardization, for the duration of a titration-curve determination. The following solutions were used for standardization, all pH values being for 40°. (I) 0·1 N-HCl, pH 1·08 (Hitchcock, 1931); (II) 0·05 N potassium hydrogen phthalate, pH 4·03 (Bates, 1948); (III) 0·1 N-KOH, pH 12·38.

The value for 0·1 N-KOH is the mean of measurements obtained in this laboratory.

Titration apparatus. Direct titration of gelatin solutions has been used, rather than the method of Hitchcock (1931) and Ames (1952), which involved addition of quantities of acid or alkali to a series of gelatin solutions. For each gelatin, the titrations with 0·2 N-HCl and with 0·2 N-KOH, were carried on side by side in two vessels (Fig. 1) at 40±0·1°, and were preceded by corresponding blank titrations of distilled water.

Each titration vessel carried two 'Alkacid' electrodes, (*A*), which were connected through a four-position selector switch to the pH meter. The saturated calomel electrodes (*B*) were connected in parallel to the same meter. Readings were taken with all four electrodes throughout the experiments; this prevented failure of one electrode from spoiling an experiment, or more usually, enabled the electrodes giving the more reproducible values, for the pH-standardization solutions, to be selected in calculating the results.

A stream of N_2 was passed through (*D*) to keep the solutions free of CO_2. The same tube was used in conjunction with the two-way tap (*E*) for withdrawing solutions or distilled water. The burette jet (*C*) was an extension fitted to a standard 10 ml. automatic burette by rubber tubing. A funnel replaced the burette when adding standardizing solutions or distilled water.

Titration procedure

Electrode standardization. The four electrodes were standardized using solution II (pH 4·03) and they were then checked by measuring the pH of the following cycle of solutions I, II, III, II, I. If any electrode showed irregular behaviour, it was removed and replaced by another electrode. After completing the titrations, the electrodes were finally checked with solution II and either I or III. The value obtained, if within 0·02 pH unit of the original value, was regarded as indicating correct functioning of an electrode throughout the titration.

Blank titration. 25 ml. of distilled water were added to each titration vessel. The stream of N_2 removed dissolved CO_2 in 10–15 min., before the commencement of the titration, raising the pH to 7·0±0·3. Additions of 0·2 N-HCl were then made to one titration vessel, and of 0·2 N-NaOH to the second. The amounts were such as to make the total added follow the steps 1, 2, 3, 5, 10 and 25 ml. After each addition, 1 min. was allowed for stirring, and the pH was then measured. The last addition brought the concentration of the reagent in each vessel to 0·1 N, and the pH was compared with that obtained during standardizing with solutions I and III. pH discrepancies of ±0·02 with the acid, and ±0·05 with the alkali were the maximum permitted divergences.

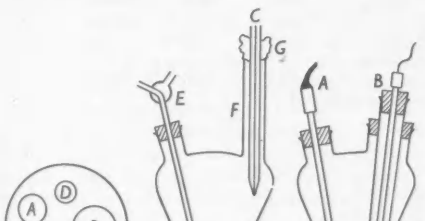

Fig. 1. Diagrammatic plan and sections of titration vessel. *A*, Doran 'Alkacid' glass electrode; *B*, Cambridge saturated calomel electrode; *C*, capillary tube extension to burette jet, surrounded by *G*, a loose plug of cotton wool; *D*, delivery tube; *E*, two-way tap; *F*, neck of vessel used for clamping.

Gelatin titration. The gelatin titrations were then performed using 25 ml. of the gelatin solution in each vessel. The volumes of acid or alkali added were regulated to produce increments of the order of 0·2–0·4 in the pH of the solutions. The limits of titration were set at pH's 1·5 and 12, since beyond these points the differences in pH between the solutions with and without gelatin were so small that the errors in the measurement of pH rendered the results inaccurate. Occasional difficulty with frothing caused by the N_2 stirring was overcome by the addition of minute amounts of *sec.*-octanol.

Back titration. After completion of the two gelatin titrations, the contents of each flask were back-titrated by addition of a volume of the complementary reagent equal to the volume of the titrating reagent already added. In the absence of gelatin decomposition, the pH values of the resulting solutions were within ±0·02 pH unit of the original values. An added indication of decomposition during titration is given by drift of pH values under the extreme acid or alkaline conditions. No drift was observed with the gelatins used.

Calculation of results

The method of calculation of the combined hydrogen ions as the pH is lowered, and of hydroxyl ions to neutralize the hydrogen ions released by the protein when the pH is raised, is essentially that given by Cohn & Edsall (1943) where earlier work is referred to in detail. The assumption is made that the relation between pH and the concentrations of hydrogen or hydroxyl ions for the same total concentration of acid or alkali is not affected by the presence of protein. In a solution of HCl of C_1 equivalents/l., the hydrogen ion concentration $[H^+]$ will be C_1 moles/l. Then it is assumed that

$$\mathrm{pH} = -\log \alpha\,[H^+], \tag{1}$$

where α is a function of C_1 only (T, P constant throughout).

$$\mathrm{pH}_1 = -\log \alpha C_1. \tag{2}$$

In the presence of protein in this same solution, $[H^+]$ becomes C_2, $C_1 - C_2$ moles of H^+ having combined with protein. In these conditions it is assumed that

$$\mathrm{pH}_2 = -\log \alpha C_2. \tag{3}$$

From (2) and (3)

$$\log C_2/C_1 = \mathrm{pH}_1 - \mathrm{pH}_2. \tag{4}$$

Similarly, for (OH') ions,

$$\log C_2/C_1 = \mathrm{pH}_2 - \mathrm{pH}_1. \tag{5}$$

The particular application of this treatment to the data of the present method is given below.

First, from the blank titration on water alone, $\log [H^+]$ and $\log [OH']$ are plotted against pH, giving straight lines. From these graphs are read off the values of pH which would be obtained, if the quantities of reagents added to the gelatin solutions were added to the same volume of water.

The values for combined ions are obtained from equations (6) which are derived from equations (4) and (5), the units being m-moles/g. gelatin.

$$\left.\begin{aligned}&\text{Acid titration:}\\&\text{combined } H^+ = \frac{v}{5g}\{1-\text{antilog}\,(pH_w - pH_g)\};\\&\text{Alkali titration:}\\&\text{combined } OH' = \frac{v}{5g}\{1-\text{antilog}\,(pH_g - pH_w)\};\end{aligned}\right\} \quad (6)$$

where v is the volume of 0·2N reagent added to 25 ml. solution; g is the weight in g. of anhydrous ash-free gelatin in the original 25 ml. of solution; pH_g is the pH of the 25 ml. of solution after the addition of v ml. of reagent; pH_w is the pH of 25 ml. of water after addition of v ml. of reagent. The combined hydrogen and hydroxyl ions are then plotted as positive and negative ordinates, using pH values as abscissae.

RESULTS AND INTERPRETATION

The resultant curve for gelatin made by the alkaline process is given in Fig. 2, where the zero of the combined ion scale is set at the isoionic point of the gelatin. Since the gelatin used had been de-ionized it was isoelectric and isoionic at the start of the titration. The position of the zero point is of no significance, however, and may be determined by the arbitrary pH of the original solution, even when this does not represent the isoionic point. The combined ion scale may be considered, after Cohn & Edsall (1943), to show the number of protons lost with increase of pH from the state of maximum positive charge at pH 1·5 or less. In the curve for the acid-processed gelatin (Fig. 3), the zero of the combined ion scale is set near pH 4, the starting point of the titrations, whereas the isoelectric and isoionic points are at pH 9·1. The difference between these two curves has been shown by Ames (1952) to be due to the conversion, by alkaline processing, of non-ionizable amide groups attached to dicarboxylic acid residues, into ionizable carboxyl groups.

Cannan (1942) has analysed the titration curves of proteins into regions characteristic of the titration of the ionizable groups present. Cannan's criteria applied to the groups present in gelatin are as follows: (*a*) pH 1·5–6, carboxyl groups; (*b*) pH 6–8·5, α-amino and imidazole groups; (*c*) pH 8·5–11·5, ε-amino groups. The values in the first line of Table 2 are obtained by analysis of the curve of alkali-processed gelatin by these criteria. Since at the isoelectric point the net molecular charge is zero, it follows that the number of carboxyl groups titrating from pH 1·5 to this point must equal the total number of charged basic groups in the molecule (Cohn & Edsall, 1943). As the only basic group not estimated directly by the curve is the guanidino group, the amount of this is calculated by difference, and is included in Table 2. Also included are values for α-amino groups calculated on a basis of a linear molecule of molecular weight 60 000; and for residual amide groups calculated as follows. The isoelectric point, pI, for this gelatin is 4·92, which is 0·10 pH unit higher than the value of pH 4·82 which is the lowest found from observations on many alkali-processed gelatins, and which may be assumed to correspond to zero amide groups. This increase must be due to the inability of a number of the carboxyl groups to ionize in the region of pH 1·5–4·8, owing to blocking by amide groups.

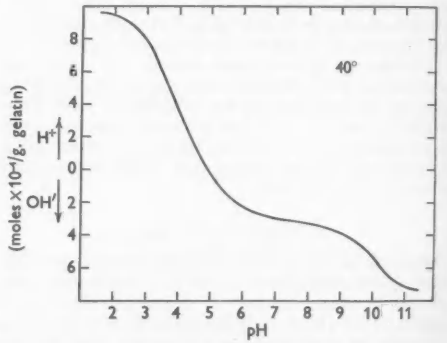

Fig. 2. Titration curve of alkali-processed gelatin, pI 4·92. This curve is based on the mean values from the five experiments summarized in Table 1. Individual points were too many to show, but cf. Fig. 3.

Table 1. *Analysis of five experimental titration curves of the same alkali-processed gelatin*

All values in m-moles/g. anhydrous ash-free gelatin.

Group	Maximum value	Minimum value	Range	Mean value
Carboxyl	1·29	1·20	0·09	1·23
α-Amino + imidazole	0·07	0·06	0·01	0·06
ε-Amino + phenolic hydroxyl	0·43	0·40	0·03	0·42

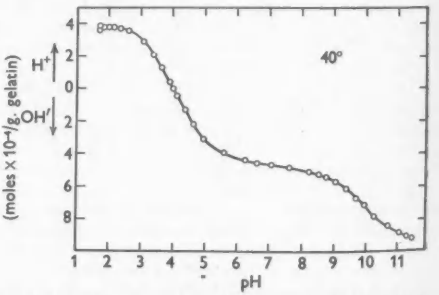

Fig. 3. Titration curve for acid-processed gelatin, pI 9·1.

Table 2. *Analysis of dissociable groups in gelatin*

All quantities in m-moles/g. of anhydrous ash-free gelatin.

	Ionizable groups					
Method of analysis	Free carboxyl + amide	Total carboxyl	ε-Amino including phenolic hydroxyl	Imidazole + α-amino	− α-Amino* = imidazole	Guanidino
From commercial alkali-processed gelatin						
Titration curve after Cannan (1942)	1·19 + 0·03†	1·22	0·39	0·12	− 0·02 = 0·10	0·44†
Chromatography after Moore & Stein by Eastoe (private communication)	1·246 + 0·052	1·298	0·41‡ + 0·015§ = 0·42(5)	.	. 0·05	0·48
Titration curve by revised criteria	1·23 + 0·03†	1·26	0·42	0·06	− 0·02 = 0·04	0·48†
Titration curve of Hitchcock (1931) by revised criteria	1·26 .	1·26	0·6 (0·48)‖	0·07	− 0·02 = 0·05	0·30† (0·41)‖
Titration curve of commercial acid-processed gelatin	0·85 + 0·35†	1·20	0·42	0·06	− 0·02 = 0·04	0·49†

* Calculated on the assumption of a linear polypeptide of molecular weight 60000 (Courts, 1953).
† Calculated from the titration curve, using p*I*.
‡ Lysine plus hydroxylysine plus ornithine.
§ Tyrosine.
‖ Values for Hitchcock's later batch.

As the rest of the molecule remains the same, the increase may be considered as equivalent to adding an amount of alkali sufficient to combine with the same number of carboxyl groups to the gelatin of p*I* 4·82 and thus to increase its pH from this figure to pH 4·92. This amount of alkali and hence the quantity of amide groups concerned can be determined from the titration curve of the gelatin of p*I* 4·82. The titration curve of this material in the region above pH 3 will be indistinguishable from that of the gelatin under consideration, of p*I* 4·92, and thus the curve in Fig. 2 may be used, giving the value 0·03 m-moles.

The second line of Table 2 consists of the comparable values derived by ion-exchange chromatography of hydrolysed gelatin by the method of Moore & Stein (1951) by Eastoe (private communication). It will be seen at once that there is, on the whole, fairly good agreement between the values obtained, except in the case of histidine. In order to resolve this discrepancy, it has been necessary to reconsider the criteria for the analysis of the titration curve.

First, the shape of the curve from pH 1·5 to 6 cannot be accounted for by assigning a single p*K* value to all the carboxyl groups. It is, however, possible to reproduce the experimental curve within the experimental accuracy by the use of two p*K* values, pK_1, pK_2, corresponding to groups present to the extent of A and $B \times 10^4$ mols/g. protein, and by use of the relation

$$\log [COO^-]/[COOH] = pH - pK,$$

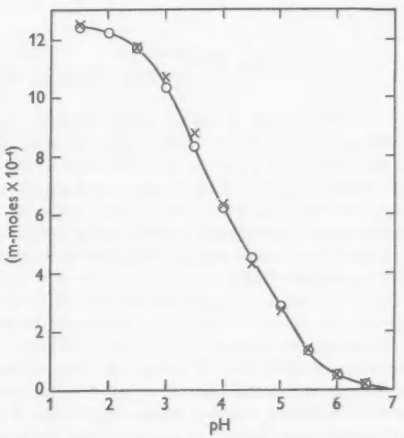

Fig. 4. Titration curve of carboxyl groups in alkali-processed gelatin. The points calculated for a mixture of 0·72 and 0·53 m-moles of acids of p*K* 3·4 and 5·0, respectively, are shown as ○, with a curve drawn through them. The experimental points for 1 g. of anhydrous ash-free gelatin are shown as ×.

which is sufficiently accurate for the present purpose. By inspection and trial the values of A, B, pK_1 and pK_2 may be adjusted to give a calculated curve in excellent agreement with the experimental one. This has been done in Fig. 4, where pK_1 = 3·4, pK_2 = 5·0, A = 7·2, B = 5·3. It is not suggested that these values are of more than empirical significance

since the curves could readily be fitted with three or more pK values. Whatever choice was made must, however, leave some 3 % of the total carboxyl titration to occur above pH 6. The use of a revised limit of pH 6·5 reduces this to less than 1 % giving a titration above pH 6·5 almost equal to that expected for α-amino and histidine below pH 6·5. The limit may not apply for other proteins unless the ratio of histidine to dicarboxylic acid residues is small, as in gelatin. Where the histidine content is comparable with that of dicarboxylic acids the 'tail' of the histidine titration below pH 6·5 would become significant.

Secondly, collagen and its derived protein gelatin are distinguished from other proteins in that they contain relatively large amounts of hydroxylysine. This has been shown by Van Slyke, Hiller, Macfadyen, Hastings & Klemperer (1940) to have pK 9·50 for its ε-amino group, compared with the figure of 10·3 for lysine. Also, whereas the heat of ionization ΔH, of the carboxyl group is practically zero, (Cohn & Edsall, 1943) and thus the pK is not temperature-dependent, the converse is true for the ε-amino groups. Cohn & Edsall (1943) give a value of $\Delta H = 10\text{–}12 \times 10^3$ cal./mole for the ε-amino groups. Using the relation

$$\Delta H = RT^2 \frac{\mathrm{d} \ln K}{\mathrm{d}T},$$

with $T = 300°$ K as a mean value for the range 20–40° c, (d/dT) p$K = -0{\cdot}02(6)$. For a temperature rise from 20 to 40°, the pK will decrease by 0·5 for both lysine and hydroxylysine residues, giving values of 9·8 and 9·0 at 40°. This difference in temperature dependence between the acidic and basic groups in amino acids was first recorded by Birch & Harris (1930).

While Cannan's range of pH 8·5–11·5 would exclude only about 10 % of the hydroxylysine at 20°, it excludes over 25 % at 40°, so that it is necessary to shift the pH range used according to the temperature. At 40°, the range will become pH 8·0–11·5, and this will mean that about 1 % of the lysine and 10 % of the hydroxylysine will titrate below this range corresponding to 0·01 m-mole/g., but this will in any case be compensated by *ca.* 15 % of the histidine and α-amino groups, titrating above pH 8·0.

Using these new criteria, namely carboxyl pH 1·5–6·5, α-amino plus imidazole pH 6·5–8·0, ε-amino pH 8·0–11·5, and the same methods as before for amide and guanidino groups, gives line three of Table 2. The maximum inaccuracy of the individual titration curve appears to be ± 5 % at the extreme ends; and for mean values from five separately determined curves, ± 2 % might be expected. This is illustrated in Table 1.

DISCUSSION

When allowance is made for the difference in temperature, the agreement between the results for the alkali-processed gelatin and those for a similar gelatin obtained by Ames (1952) is excellent. The difference between the present curve and that of Ames for acid-processed gelatins lies entirely in the estimated amount of carboxyl groups present. Ames does not specify the isoelectric point of his material more closely than 'about neutrality' whereas the present material had p$I = 9{\cdot}1$; it is of interest that by applying the converse of the method used earlier to determine the amide content from the isoelectric point, the calculated values are p$I = 7{\cdot}5$ for Ames' material, and p$I = 9{\cdot}1$ for the present material. These comparisons can be justified only if the total amino acid composition of both materials is closely similar to that of the lime-processed hide gelatin used in the present work. This is substantiated by the titration values for the basic groups, and by the estimation of the amide groups and their addition to the titratable carboxyl groups to give the total value for dicarboxylic acids. The agreement with the values, calculated by the revised criteria, for the curve obtained by Hitchcock (1931) for an Eastman Kodak gelatin is excellent, except in that his ratio of ε-amino groups to guanidino groups is considerably different, as is apparent from the shape of the high pH portion of his curve. As Hitchcock's work appears to have been carefully performed, the most probable explanation is a difference in the composition of the gelatin used. Albanese (1940) recorded a high 'lysine'-value for an Eastman Kodak gelatin, although the arginine figure was not correspondingly reduced.

Since the total number of basic groups in Hitchcock's gelatin remained unchanged, as shown by the pI 4·84, a possible explanation is that considerable arginine → ornithine conversion had occurred. The conversion of a guanidino group into an amino group is known to occur to a slight extent during the alkaline pre-treatment of collagen (Bowes & Kenten, 1948), but a different order of change would be required to account for the results. Hitchcock noted the discrepancy between his work and earlier results reviewed by Cohn (1925). He recorded that another batch of gelatin from the same source gave results in better agreement with the earlier work. The values for this second batch, in so far as they are available, are given in brackets with the comparable figures for the main work in line 4 of Table 2.

Another possibility, suggested by Ames (1952), is that the anomalous gelatin used by Hitchcock was decomposing under alkaline titration conditions; such decomposition can produce this type of curve. The absence of a titration back to the zero point, as

used in the present method, leaves this point uncertain.

The agreement between the titration curve estimation by the new criteria (Table 2, line 3) and the chromatographic estimation (Table 2, line 2) for the alkali-processed gelatin is so close as to be within the experimental error of the former. It is possible that there is a real discrepancy in the values for lysine, but this point is being further investigated. The correction of the imidazole figure by a reduction of approximately 50 % automatically adjusts the arginine figure by the same absolute, but proportionately smaller, amount, and brings this latter value into good agreement.

SUMMARY

1. A rapid method of determining the titration curve of gelatins is described and the accuracy indicated.

2. Revised criteria for the interpretation of the titration curve are presented.

3. The anomalous values for the histidine content of gelatin by titration curve analysis are brought into agreement with those from other methods.

4. The whole of the analytical data obtainable by use of the titration curve and isoelectric point are shown to be in agreement with those from chromatographic analysis.

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Oxidative Phosphorylation in Insect Sarcosomes

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The unique structure of the indirect flight muscles of certain insects belonging to the natural orders Hymenoptera and Diptera has been known for many years. Aubert (1853) was the first to describe the unusually large and abundant granules, later named sarcosomes by Retzius (1890), which are a characteristic feature of these muscles.

Keilin (1925) stated that 'among all the organisms examined the highest concentration of cytochrome is found in the thoracic muscles of flying insects'. On the other hand, little cytochrome was found in the corresponding muscles of wingless insects. Keilin suggested that the high concentration in the thoracic muscle of flying insects had a connexion with their unusually high activity. The wing muscle of the housefly, for example, is capable of producing more than 300 separate contractions per second (Marey, 1874).

Recently, Williams and his colleagues have shown that the cytochrome in the thoracic muscles of *Drosophila funebris* and the blowfly *Phormia regina* is contained in the sarcosomes (Watanabe & Williams, 1951, 1953; Levenbook, 1953). In this respect and in their chemical composition and enzymic content, the insect sarcosomes resemble mitochondria from vertebrate tissues. Nevertheless, until 1953, oxidative phosphorylation, which is a characteristic property of mitochondria and sarcosomes of vertebrate tissues, had not been demonstrated in insect sarcosomes. Sacktor (1953) reported that although sarcosomes isolated from the housefly *Musca domestica* L. oxidized α-ketoglutarate (α-oxoglutarate) rapidly, he was unable to show oxidative phosphorylation. He found that this was not due to the adenosinetriphosphatase (ATPase) activity of the sarcosomes, since the added hexokinase and glucose were able to compete successfully with the ATPase for added ATP. As already reported briefly (Lewis & Slater, 1953), we were able to demonstrate oxidative phosphorylation in sarcosomes isolated from the blowfly *Calliphora erythrocephala*. At about the same time, Watanabe & Williams (1953) quoted a personal communication from Sacktor stating that he had demonstrated

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oxidative phosphorylation, 'but only in the presence of proteinaceous media'.

The present paper describes our previously reported experiments in greater detail, and also includes additional experiments suggested by the work of Watanabe & Williams (1953). Since the completion of our experiments, Sacktor (1954) has published an account of his work referred to by Watanabe & Williams (1953). A comparison of his findings with ours will also be given in this paper.

METHODS

Isolation of sarcosomes

The flies (*C. erythrocephala*) were bred at 22° according to the technique described by Tate (1948). On emergence, the adults to be used in the experiments were maintained on a diet of glucose and water, and batches of mixed sexes were removed at intervals.

The method of isolating the sarcosomes was similar to that described by Watanabe & Williams (1951). The flies were immobilized by cooling at 0°, and placed on a cold glass plate. The head and abdomen were removed and the thoraces placed in an ice-cold mortar. When the required number of thoraces had been obtained they were crushed gently with a pestle (without sand) in about 1 ml. of suspension medium for each 20 thoraces. The suspension was then filtered through six layers of muslin, previously saturated with suspension medium, into a centrifuge tube immersed in an ice bath. The muscle pulp was washed with several portions of cold suspension medium using a total of 1 ml. for each thorax. The suspension was centrifuged at 0° for 4 min. at 200 *g* and the supernatant again centrifuged for 8 min. at 1650 *g*. The sedimented sarcosomes were resuspended in isolation medium to make a total volume of 0·8 ml. for each 20 thoraces used.

Several different isolation media were used in this study. The pH of all was 7·4, except the medium of pH 7·0 described by Watanabe & Williams (1953). Because of the high dilution of the sarcosomal suspension before centrifuging, the sedimented sarcosomes contained comparatively little sarcoplasm and further washing was not considered necessary. In trial experiments, the effect of washing the sarcosomes on the phosphorylation ratio was variable, decreasing it in one experiment, increasing it in another and having no effect in a third.

Measurement of activity of α-ketoglutaric oxidase system and of accompanying phosphorylation

The activity of the sarcosomes was measured by the procedure previously described (Slater, 1953; Slater & Holton, 1953). Except where stated otherwise, the reaction mixture used was that found optimum for heart-muscle sarcosomes and differed only slightly from that described as 'standard conditions' by Slater & Holton (1954). Usually 1–1·5 mg. sarcosomal protein and 3–4 μmoles α-ketoglutarate were used. At least 100 units of yeast hexokinase of specific activity (Berger, Slein, Colowick & Cori, 1946) 1550 were added for each mg. of sarcosomal protein. The reaction period was either 35 or 45 min. at 25°. Even though the reaction mixture contained 0·01 M malonate to inhibit succinic dehydrogenase, the O_2 uptake (μatoms) greatly exceeded the α-ketoglutarate (α-Kg) disappearance (μmoles). The ratio (ΔO:Δα-Kg) varied between 1·3 and 4·1, and was usually above 2. Although some of the excess O_2 uptake may be due to traces of residual substrates in the sarcosomal preparation or in the hexokinase preparation (the ratio of the rate of O_2 uptake in the absence of α-ketoglutarate to that in its presence was between 0·04 and 0·32, with a mean value 0·21, in five experiments), it is probably mainly due to further oxidation of the succinate formed from the α-ketoglutarate.

The activity of the α-ketoglutaric oxidase system is expressed as Q_{O_2}, but refers only to that fraction of the observed O_2 uptake concerned in the oxidation of α-ketoglutarate to succinate. It was calculated from the expression $Q_{O_2} = \frac{11{\cdot}2 \times 60 \times \Delta\alpha\text{-Kg}}{wt}$ μl. O_2/hr./mg., where w (mg.) is the amount of sarcosomal protein and t (min.) the time of the experiment. The Q_{O_2} was found to vary with the α-ketoglutarate concentration (see p. 213). A correction curve was constructed to convert the observed Q_{O_2} obtained with between 0·0025 and 0·006 M α-ketoglutarate into a standard concentration (0·005 M). The correction factor varied between 1·16 with 0·0025 M α-ketoglutarate and 0·94 with 0·006 M α-ketoglutarate. The corrected activity is referred to as Q_{O_2} (corr.).

The amount of phosphate esterified (Δ esterified P) = ΔHMP (hexose monophosphate) + Δ~P (reactive energy-rich phosphate groups of adenosine diphosphate (ADP) and adenosine triphosphate (ATP)). The myokinase activity, as measured by −Δ~P, was about the same as in heart sarcosomes (Slater, 1953). The yield of phosphorylation is expressed either as P:O (atoms of phosphorus esterified per atom of oxygen consumed) or as P:α-Kg (atoms of phosphorus esterified per molecule of α-ketoglutarate disappearing). The true phosphorylation ratio for the oxidation of α-ketoglutarate to succinate will lie between these two values. When ΔO:Δα-Kg is less than 2, P:α-Kg will be closer to the true value, since only slight phosphorylation accompanied the oxidation of succinate. The oxidation of endogenous substrate was also accompanied by only slight phosphorylation; the P:O ratio varied between 0·10 and 0·36, with a mean of 0·20, in four experiments. When the ΔO:Δα-Kg ratio is much greater than 2, the P:α-Kg ratio becomes of little value.

RESULTS

Effect of age of blowfly

The phosphorylation ratios varied considerably from batch to batch of flies. Studies of individual batches at different ages (Table 1) showed that the

Table 1. *Effect of age of fly on phosphorylation ratios*

Isolation media: 0·36 M sucrose containing 0·005 M phosphate for batch *A*; 0·32 M sucrose containing 0·01 M ethylenediaminetetraacetic acid for batch *B*.

Batch	Age (days)	Q_{O_2} (corr.)	P:O	P:α-Kg
A	1–7	18·6	0·57	1·29
	6–12	12·6	0·72	1·56
	9–15	12·8	0·94	2·95
B	1–3	11·5	0·88	1·48
	8–10	9·4	1·79	4·81
	15–17	12·0	1·72	3·28

ratios were distinctly lower with young flies (less than 1 week from emergence). Watanabe & Williams (1953) have shown that the average diameter of the sarcosomes of *Drosophila funebris* and of the blowfly *Phormia regina* increases rapidly during the first week of life and then remains constant.

Effects of varying method of isolating sarcosomes

Time of standing of thoraces. In studies of oxidative phosphorylation, it is usually considered desirable to remove the tissue from the animal as soon as possible after death. In the case of blowflies, where 20–70 individuals are used in any one sarcosomal preparation, this is not convenient. In any case, the situation is not quite comparable, since the wing movements of the thorax are still obvious some time after dissection. Nevertheless, it was considered desirable to test whether the time of standing of the isolated thoraces, at or just below room temperature, had any effect on the phosphorylation ratios. Such an experiment is summarized in Table 2, which shows that prolonged standing (35 min.) caused definitely reduced ratios. In all the other experiments described in this paper, the time of standing of the thoraces did not exceed 5 min.

Degree of grinding of thoraces. A very mild crushing of the muscle with a pestle is sufficient to liberate the sarcosomes from the fibre. Even grinding for only 1 min. without sand caused a considerable decline in the phosphorylation ratios (P:O = 0·58, compared with 0·94; P:α-Kg = 1·69, compared with 2·95).

Tonicity of the isolation medium. Mr T. D. Iles of the Department of Zoology, University of Cambridge, kindly measured the freezing-point depression of a sample of blowfly haemolymph for us. The value obtained was 0·72°, which is considerably greater than for rat serum (0·51°, Conway & McCormack, 1953). From data in the International Critical Tables (1928), it was calculated that 0·36 M sucrose would be isotonic with haemolymph. Assuming that blowfly thoracic muscle is isotonic with haemolymph, it appeared logical to use this medium to isolate the sarcosomes. The effect of varying the tonicity of the isolation medium is shown in Fig. 1. It can be seen that the highest phosphorylation ratios are obtained in the region of the tonicity of the haemolymph. It is noteworthy that the preparation isolated under the most hypotonic conditions oxidized α-ketoglutarate most rapidly, but gave comparatively low phosphorylation ratios.

The low ratio obtained by the use of 0·56 M sucrose containing 0·01 M ethylenediaminetetraacetic acid as isolation medium is partly due to the high concentration of sucrose in the reaction mixture. A control experiment with another preparation showed that the inclusion of this additional sucrose in the reaction mixture lowered the P:O ratio of sarcosomes isolated with 0·36 M sucrose from 1·03 to 0·79.

In most of the experiments reported in this paper the tonicity of the isolation medium was 0·36 osmolar; in a few it was 0·20 osmolar, which would be expected from Fig. 1 to give a P:O ratio about 10 % lower than that obtained with the optimum isolation medium.

Table 2. *Effect of time of standing of isolated thoraces on phosphorylation ratios*

Isolation medium: 0·20 M sucrose containing 0·002 M ethylenediaminetetraacetic acid. The time of standing refers to the time between removing the head and abdomen of the fly and commencing the isolation procedure. The preparations were made simultaneously from the same batch of flies. Concn. of α-ketoglutarate, 0·003 M.

Time of standing (min.)	Q_{O_2}	P:O	P:α-Kg
0	9·5	0·83	1·88
5	11·9	0·86	1·68
35	11·4	0·65	1·39

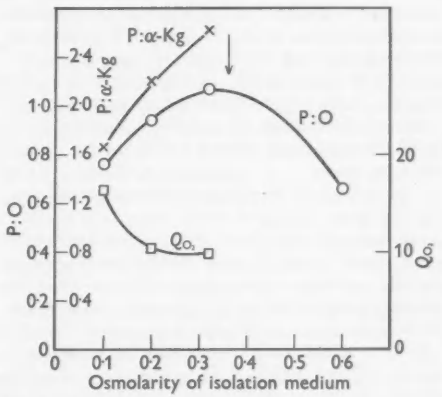

Fig. 1. Effect of varying the tonicity of the isolation medium on the activity of the α-ketoglutaric oxidase system and on the phosphorylation ratios. The isolation media contained 0·01 M ethylenediaminetetraacetic acid and 0·06, 0·16, 0·28 or 0·56 M sucrose, respectively. The four preparations were made simultaneously from the same batch of blowflies, using 20 blowflies to each preparation. All preparations were tested in the same reaction mixture, except that the sucrose concentration varied between 0·009 and 0·084 M. The arrow gives the osmolarity of sucrose with the same freezing-point depression as blowfly haemolymph. (The osmolarity is defined as the total concentration of all species of solute in the isolation medium (g.moles or g.ions/l.), i.e. osmolarity = [sucrose] + 4[ethylenediaminetetraacetate], since at pH 7·4, the chelating agent is present largely as the tribasic anion.) Concn. of α-ketoglutarate, 0·003 M.

Composition of isolation media. (1) *Ethylenediaminetetraacetic acid.* The effect of including ethylenediaminetetraacetic acid in the isolation medium is shown in Table 3. It caused a statistically significant ($P = 0{\cdot}05$) decrease in the activity of the α-ketoglutaric oxidase system. This is in contrast to the behaviour of rat-heart sarcosomes where the inclusion of ethylenediaminetetraacetic acid in the isolation medium considerably increased the activity of the α-ketoglutaric oxidase system (Slater & Cleland, 1953*b*). However, in agreement with the experience with rat heart, the presence of ethylenediaminetetraacetic acid in the suspension medium stabilized the α-ketoglutaric oxidase system in blowfly sarcosomes (Table 4). In contrast with the behaviour of heart sarcosomes, the phosphorylation ratios were more affected than the rate of oxidation, by incubation of the preparation in the absence of the chelating agent. It should be noted that except in some experiments described in Tables 5 and 7, ethylenediaminetetraacetic acid was always present in the reaction mixture.

The other differences in Table 3 are not statistically significant ($P > 0{\cdot}05$).

(2) *Saline.* Only one preparation was made with saline (0·173 M-KCl containing 0·01 M ethylenediaminetetraacetic acid). This had a very high Q_{O_2} (50, compared with 18·7 given by sarcosomes isolated in 0·36 M sucrose containing 0·005 M phosphate using the same batch of flies on the same day) and a low phosphorylation ratio (P:O = 0·61, P:α-Kg = 0·73, compared with 0·90 and 1·70, respectively, for the sucrose preparation). Sacktor (1954) has also found lower phosphorylation ratios using saline isolation media.

(3) *Albumin.* Watanabe & Williams (1953) have found that a solution of 2·5% bovine-plasma albumin in 0·16 M phosphate, pH 7·0, was the optimum medium for the preservation of the microscopic appearance of isolated sarcosomes. Accordingly, this medium was compared with sucrose in the experiment summarized in Table 5. Since the addition to the reaction mixture of the sarcosomes suspended in this medium introduced a considerable concentration of albumin (0·37%) and additional phosphate (0·024 M) and lowered the pH somewhat, the comparison was made by adding the same concentration of albumin and phosphate to the reaction mixture used for measuring the activity of the sarcosomes isolated in sucrose. As will be shown later, the addition of albumin to the reaction mixture markedly stimulated the oxidation of α-ketoglutarate. However, it is clear from Table 5 that the isolation medium of Watanabe & Williams is not as suitable as sucrose for obtaining maximum phosphorylation ratios. In fact, the former preparation resembles that obtained with potassium chloride.

The addition of 2·5% albumin to the usual sucrose medium was also tried in the experiment shown in Table 6. The addition increased the phosphorylation ratios slightly, but the increase was well within the range of variation between individual preparations and was no more than that often obtained by adding ethylenediaminetetraacetic acid to the sucrose.

Table 3. *Comparison of preparations made with and without ethylenediaminetetraacetic acid*

The values given are mean ± standard error of the mean. Four preparations with abnormally low phosphorylation ratios (P:α-Kg less than 1) have been omitted from this series.

	Isolation medium	
	0·36 M Sucrose + 0·005 M phosphate	0·32 M Sucrose + 0·01 M ethylenediaminetetraacetic acid
No. of preparations	7	9
Age of flies (days)	10·2	8·6
Q_{O_2} (corr.)	17·35 ± 1·95	12·9 ± 0·76
P:O	1·00 ± 0·13	1·19 ± 0·15
ΔO:Δα-Kg	2·64 ± 0·28	2·04 ± 0·13

Table 4. *Effect of incubating sarcosomes*

The incubation procedure was the same as that described by Slater & Cleland (1953*b*).

	Isolation medium	
	0·36 M Sucrose + 0·005 M phosphate	0·32 M Sucrose + 0·01 M ethylenediaminetetraacetic acid
Not incubated		
Q_{O_2}	18·7	7·4
P:O	0·90	0·88
P:α-Kg	1·70	1·48
Incubated 15 min. at 25°		
Q_{O_2}	14·6	7·2
P:O	0·57	0·83
P:α-Kg	0·69	1·34

Table 5. *Comparison of aqueous solutions of bovine-plasma albumin and of sucrose as isolation media*

The reaction mixture included 0·37% albumin and 0·024 M phosphate buffer, pH 7·0, in addition to 'standard conditions'.

	Isolation medium	
	0·16 M Phosphate, pH 7·0 + 2·5% albumin	0·36 M Sucrose + 0·005 M phosphate, pH 7·4
Q_{O_2}	46*, 47	25*, 27
P:O	0·82*, 0·78	1·00*, 0·95
P:α-Kg	0·99*, 0·93	1·49*, 1·39

* Measured without ethylenediaminetetraacetic acid in the reaction mixture.

Table 6. *Effect of adding albumin to isotonic sucrose isolation medium*

The isolation medium was 0·36 M sucrose + 0·005 M phosphate, pH 7·4, with or without 2·5% albumin. The reaction mixture included 0·37% albumin in addition to 'standard conditions'.

	Isolation medium	
	With albumin	Without albumin
Q_{O_2}	33	30
P:O	1·39	1·24
P:α-Kg	3·14	2·55

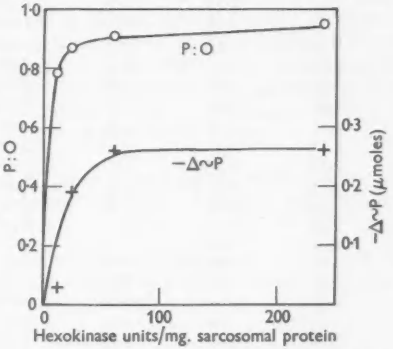

Fig. 2 (*a*). Effect of varying the amount of hexokinase on the P:O ratio and on Δ∼P. Sarcosomes isolated in 0·20 M sucrose containing 0·002 M ethylenediaminetetraacetic acid.

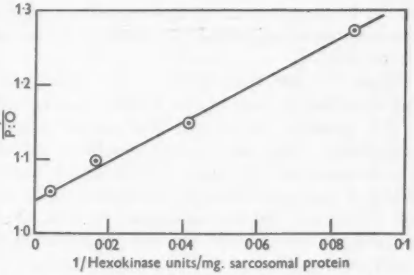

Fig. 2. (*b*). Fig. 2 (*a*) plotted according to procedure of Lineweaver & Burk (1934).

Effects of varying reaction mixture

Concentration of hexokinase. With any preparation containing ATPase, it is important that sufficient hexokinase should be added so that virtually all the ATP formed by oxidative phosphorylation reacts with this enzyme and none with the ATPase. The effect of varying the amount of hexokinase on the P:O ratio is shown in Fig. 2 (*a*). It can be shown (see the Appendix) that in a system in which there is competition between hexokinase and ATPase (or other ATP-consuming systems) for available ATP, the relationship between the concentration of hexokinase and the amount of ATP which reacts with it may be represented by a rectangular hyperbola. Fig. 2 (*b*) shows the same results plotted by the Lineweaver & Burk (1934) method, which converts a rectangular hyperbola into a straight line. From this line, it can be calculated that 50, 90, 95 and 99% of the ATP will react with the glucose in the presence of 2·5, 22, 47 and 246 units of hexokinase/mg. sarcosomal protein respectively, with this particular sarcosomal preparation. Provided that more than 100 units of hexokinase/mg. sarcosomal protein are used, the error due to loss of ATP by hydrolysis will be insignificant. It is interesting that the amount of hexokinase required for 50% of the ATP to react is identical with that calculated by the same method from the results of a similar experiment with rat-heart sarcosomes described by Slater & Holton (1953). Fig. 2 (*a*) shows also that the decrease of ∼P is maximum with about 70 units of hexokinase/mg. sarcosomal protein. With less hexokinase, the loss of ∼P caused by the combined action of myokinase and hexokinase (or ATPase) is partly compensated for by the building up of a steady-state concentration of ATP (cf. Slater & Holton, 1953).

Another possible source of error in measuring phosphorylation is the presence in the reaction mixture of phosphatases hydrolysing hexose monophosphate, the final product of the reaction. Direct test by the method described previously (Slater, 1953) showed, however, that no phosphatase (phosphomonoesterase) was detectable.

Ethylenediaminetetraacetic acid and albumin. Table 7 shows the effects of adding ethylenediaminetetraacetic acid and bovine plasma albumin, either separately or together, to the reaction mixture. Ethylenediaminetetraacetic acid had an appreciable effect on the activity of the α-ketoglutaric oxidase system and a considerable effect on the phosphorylation ratios when added in the absence of albumin, but little effect in the reaction mixture containing 3·7 mg. albumin/ml. (Expt. 2). Expt. 3 shows that 3×10^{-4} M ethylenediaminetetraacetic acid was insufficient for maximum phosphorylation ratios, either in the absence or in the presence of 1·2 mg. albumin/ml. Albumin itself very markedly increased the activity of the α-ketoglutaric oxidase system, without usually appreciably affecting the phosphorylation ratios. In Expt. 4, the P:O ratio was increased somewhat, while the P:α-Kg ratio was decreased. This can be explained by the decreased ΔO:Δα-Kg ratio associated with the addition of the albumin. In Expt. 5, the phosphorylation ratios were slightly decreased by the addition of albumin. In Expt. 6, they were appreciably increased.

Table 7. *Effect of including bovine plasma albumin or ethylenediaminetetraacetic acid or both in reaction mixture*

The isolation media were: 0·36 M sucrose + 0·005 M phosphate in Expts. 1, 2, 4 and 5; 0·20 M sucrose + 0·002 M ethylenediaminetetraacetic acid in Expt. 3; and 0·36 M sucrose + 0·01 M ethylenediaminetetraacetic acid in Expt. 6. The concentrations of ethylenediaminetetraacetic acid, albumin and cytochrome *c* refer to the final reaction mixture.

Expt.	Ethylenediaminetetraacetic acid (m-moles/l.)	Albumin (mg./ml.)	Cytochrome *c* (mg.*/ml.)	$-\Delta O$ (μatoms)	$-\Delta\alpha$-Kg (μmoles)	Δ Esterified P (μmoles)	P:O	P:α-Kg
1	0	0	0·6	1·29	0·45	0·57	0·44	1·26
	1·5	0	0·6	1·38	0·73	1·24	0·90	1·70
2	0	0	0·6	1·33	0·50	0·78	0·59	1·56
	1·5	0	0·6	1·61	—	1·15	0·72	—
	0	3·7	0·6	2·16	1·44	2·15	1·00	1·49
	1·5	3·7	0·6	2·37	1·62	2·24	0·95	1·39
3	0·3	0	0·6	1·77	0·76	2·36	1·33	3·10
	1·5	0	0·6	1·73	0·73	2·91	1·68	4·00
	0·3	1·2	0·6	2·67	1·31	4·31	1·62	3·30
	1·5	1·2	0·6	2·64	1·26	4·52	1·71	3·61
4	1·5	0	0·6	2·86	0·91	2·68	0·94	2·95
	1·5	3·7	0·6	3·84	2·18	4·62	1·20	2·12
	1·5	10·0	0·6	3·78	2·23	4·12	1·09	1·85
5	1·5	0	0·6	4·07	1·74	5·17	1·27	2·98
	1·5	1·2	0·6	5·00	2·53	6·46	1·29	2·56
	1·5	2·4	0·6	5·30	2·69	6·20	1·17	2·30
	1·5	3·7	0·6	5·16	2·51	6·40	1·24	2·55
	1·5	0	0	4·09	1·61	5·35	1·31	3·32
	1·5	0	3·7	3·90	1·91	4·42	1·14	2·32
	1·5	3·7	0	5·34	2·60	6·35	1·19	2·44
6	1·5	0	0·3	0·89	0·53	1·30	1·46	2·45
	1·5	1·2	0·3	1·60	0·90	2·85	1·78	3·16
	1·5	5·0	0·3	1·805	0·98	3·44	1·91	3·51
	1·5	10·0	0·3	2·06	0·89	4·10	1·99	4·60

* Calculated as cytochrome *c* containing 0·45% iron.

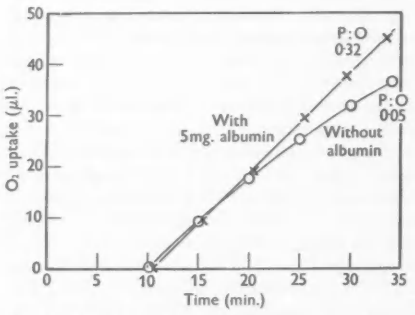

Fig. 3. Effect of bovine plasma albumin on the oxidation of succinate by blowfly sarcosomes and on the P:O ratio. Reaction mixture same as for α-ketoglutarate, except 0·01 M succinate replaced α-ketoglutarate, and fluoride and malonate were omitted. Sarcosomal protein, 0·77 mg./ml.; hexokinase, 165 units/mg. sarcosomal protein. The reaction commenced at zero time, the first manometric readings were taken at the times corresponding to zero oxygen uptake on the curves.

In Expt. 5, the same degree of stimulation was obtained with all the concentrations of albumin tested (between 1·2 and 3·7 mg./ml.). In Expt. 4, 10 mg. albumin/ml. gave the same result as 3·7 mg./ml. In Expt. 6, however, higher concentrations of albumin were appreciably more effective than 1·2 mg./ml.

Expt. 5 shows that omitting the cytochrome *c* had no effect on any of the measurements, either in the presence or in the absence of albumin. Apparently, the sarcosomes contain sufficient cytochrome *c* for optimum respiration (cf. Sacktor, 1954). A high concentration of cytochrome *c* could not substitute for the albumin; in fact, it was appreciably inhibitory to the phosphorylation (contrast the effect on rat-heart sarcosomes, Slater & Cleland, 1953*a*).

The action of albumin was also tested with succinate as substrate. Fig. 3 shows that albumin had little effect on the initial rate of oxidation of succinate, but prevented the marked fall off in the rate of oxygen uptake. It is possible that albumin promotes the oxidation of the inhibitory oxaloacetate, thereby preventing its accumulation in the system. The P:O ratio was also markedly increased by the addition of albumin.

α-Ketoglutarate concentration. It was thought possible that the activating effect of albumin was due to its ability to increase the permeability of the

sarcosomes to α-ketoglutarate. This was tested by measuring the activity, both in the absence and in the presence of albumin, at different concentrations of α-ketoglutarate (see Table 8). If the rate of penetration of α-ketoglutarate to its dehydrogenase in or on the sarcosomes were a rate-limiting step in the oxidation, it would be expected that the rate of oxidation would be increased by increasing the concentration of α-ketoglutarate. This was found to be the case. It should be noted that the rate of oxidation of α-ketoglutarate by heart sarcosomes is scarcely affected by variation of the α-ketoglutarate concentration within the range shown in Table 8 (see Slater & Holton, 1954). Further, as would be expected if there were a permeability barrier to α-ketoglutarate, the relationship between α-ketoglutarate concentration and rate of oxidation shown in Table 8 does not follow that predicted from the Michaelis & Menten (1913) theory.

Table 8. *Effect of varying the α-ketoglutarate concentration, both in the presence and absence of albumin*

Isolation medium: 0·32M sucrose + 0·01M ethylenediaminetetraacetic acid. Ratio = value in presence of albumin/value in absence of albumin.

	Albumin (mg./ml.)	α-Ketoglutarate (m-moles/l.) 2·0	4·5	9·8
−ΔO (μatoms)	0	1·02	1·33	1·87
	1·2	1·86	2·51	3·21
Ratio		1·82	1·89	1·72
−Δα-Kg	0	0·48	0·70	—
(μmoles)	1·2	1·03	1·36	—
Ratio		2·15	1·95	—
Δ Esterified P	0	1·77	2·18	3·27
(μmoles)	1·2	2·76	4·45	5·78
Ratio		1·56	2·04	1·76
P:O	0	1·73	1·64	1·75
	1·2	1·49	1·77	1·80

It seems possible then that the slow rate of oxidation of α-ketoglutarate is partly due to the slow penetration of α-ketoglutarate to its dehydrogenase. But even if this is so, the activating effect of albumin cannot be explained on this basis, since the relative effect of increasing the concentration of α-ketoglutarate was independent of the presence of albumin.

Malonate and fluoride. The effect of omitting malonate and fluoride from the reaction mixture is shown in Table 9. In Expts. 1 and 2, the omission of the malonate caused a greatly increased rate of respiration (especially in the first 15 min.), a decreased disappearance of α-ketoglutarate and a considerably increased esterification of inorganic phosphate. The stimulation of the disappearance of α-ketoglutarate by the addition of malonate is interesting. It is also shown, but to a smaller degree, in similar experiments with rat-heart sarcosomes (Table 5 of Slater & Holton, 1954). It is probably an indirect effect; the rapid oxidation of succinate and its oxidation products which occurs in the absence of malonate might suppress the oxidation of α-ketoglutarate by competition for a common intermediate, involved either in the hydrogen-transfer to oxygen or in the accompanying phosphorylation. By slowing down these reactions, malonate will promote the oxidation of α-ketoglutarate. This effect of malonate was not shown in the presence of albumin (Expts. 3 and 4 in Table 9).

The markedly different ΔO:Δα-Kg ratios obtained in the presence and absence of malonate in Expts. 1 and 2 makes it difficult to compare the respective P:O ratios. Expt. 3 shows, however, that 0·01M malonate has little if any effect on the phosphorylation associated with the oxidation of α-ketoglutarate to succinate. Doubling this concentration of malonate also had little effect (Expt. 4).

Omission of fluoride had no marked effect on the phosphorylation ratios. In Expt. 1, Table 9, the

Table 9. *Effect of malonate and fluoride*

Isolation medium: 0·36M sucrose + 0·005M phosphate in Expts. 1, 2 and 3; same medium containing 2·5% albumin in Expt. 4.

Expt.	Malonate (M)	NaF (M)	−ΔO (μatoms)	−Δα-Kg (μmoles)	ΔO:Δα-Kg	Δ Esterified P (μmoles)	P:O	P:α-Kg
1	0·01	0·04	1·61	—	—	1·15	0·72	—
	0	0·04	2·76	0·66	4·19	2·48	0·90	3·76
	0·01	0	1·52	0·60	2·54	1·40	0·92	2·33
	0	0	1·69	0·48	3·52	1·23	0·73	2·56
2	0·01	0·04	2·86	0·91	3·14	2·68	0·94	2·95
	0	0·04	4·80	0·66	7·29	4·57	0·95	6·94
	0·01	0	2·34	0·68	3·44	1·94	0·83	2·85
3*	0·01	0·04	5·16	2·51	2·06	6·40	1·24	2·55
	0	0·04	7·60	2·57	2·96	6·87	0·91	2·68
4*	0·01	0·04	5·45	2·42	2·25	7·59	1·39	3·14
	0·02	0·04	4·01	2·30	1·75	5·26	1·31	2·30

* Reaction mixture contained 3·7 mg. albumin/ml.

initial rate of oxygen uptake was considerably increased by omitting the fluoride, but it rapidly declined. This may be associated with the very low concentration of ADP which is obtained in the absence of fluoride, owing to action of myokinase and hexokinase. Fluoride is a strong inhibitor of myokinase (Kotelnikova, 1950; Barkulis & Lehninger, 1951).

Effect of time of experiment

Fig. 4 shows the results of an experiment in which one of five replicate flasks was removed and the contents deproteinized at various time intervals. The first point to be noted is that, as might be expected, the ΔO:Δα-Kg ratio increased with time, particularly during the first 10 min. The ratio after 35 min. was unusually high in this experiment. Secondly, after a rapid phase in the first 5 min., the rate of esterification of phosphate remained constant between 5 and 35 min. Probably as a consequence of the change of ΔO:Δα-Kg ratio in the first 10 min., the P:O ratio declined and the P:αKg ratio increased during this period. However, both phosphorylation ratios remained almost constant between 10 and 35 min. It seems probable, then, that no inactivation of the phosphorylating enzymes occurred in 35 min. under the conditions of this experiment. The marked fall in both the P:O and the P:α-Kg ratios between 35 and 60 min. shows, however, that a considerable inactivation occurred after 35 min.

P:O *ratios*

Fig. 5 summarizes our results with 27 blowfly sarcosomal preparations, isolated with media containing 0·20–0·36 M sucrose, with or without ethylenediaminetetraacetic acid, and measured under 'standard conditions'. The results with albumin in the reaction mixture are excluded.

The P:O ratios of the blowfly sarcosomes are much lower and show much greater variation than those of heart sarcosomes (cf. Fig. 5 of this paper with Fig. 3 of Slater & Holton, 1954).

One might expect somewhat lower P:O ratios with the blowfly sarcosomes, since a greater proportion of the oxygen uptake is associated with the oxidation of succinate to fumarate, a reaction with a low phosphorylation ratio. Nevertheless, the difference in P:O ratios is much greater than can be accounted for by the different ΔO:Δα-Kg ratios and must reflect a true difference between the phosphorylative abilities of the two preparations.

The following factors probably contribute to the variability of the P:O ratios of blowfly sarcosomes: (i) age of fly, (ii) a real difference between different batches of flies, (iii) variation of degree of crushing of thoracic muscle during the preparation of the sarcosomal fraction. It is likely that the second factor is the most important. Variation between individual blowflies is not likely to be important, since at least 20 flies were used in each preparation. It might be significant that most of the very low ratios were obtained within a few weeks during the summer. The temperature during the development of these blowflies sometimes exceeded 22°.

Sacktor's (1954) figures for the P:O ratios of housefly sarcosomes are within the range covered by Fig. 5.

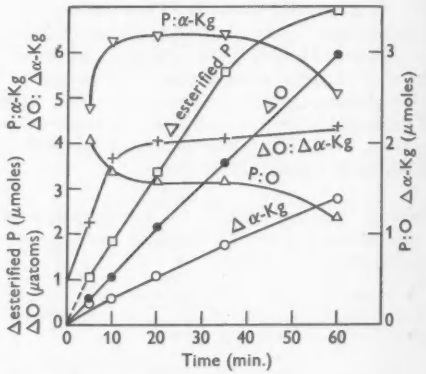

Fig. 4. Oxygen uptake, α-ketoglutarate disappearance, esterification of phosphate and the various ratios as a function of time. Sarcosomes (isolated with 0·36 M sucrose + 0·005 M phosphate), 1·33 mg. sarcosomal protein/ml.; α-ketoglutarate, 0·0045 M.

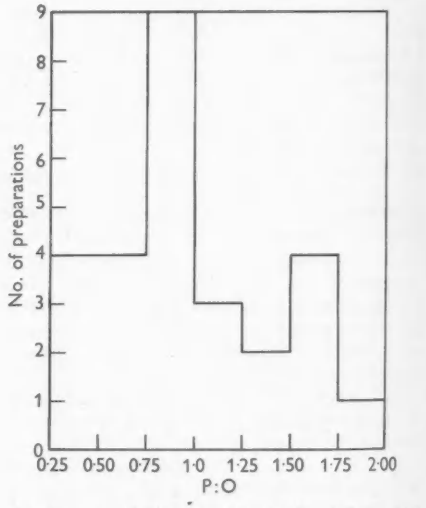

Fig. 5. Frequency distribution of P:O ratios obtained with 27 blowfly sarcosomal preparations.

DISCUSSION

Activity of the α-ketoglutaric oxidase system

The oxidation of α-ketoglutarate by the blowfly sarcosomes is slow, the average Q_{O_2} of preparations isolated in sucrose–ethylenediaminetetraacetic acid being only 13. This is to be compared with 80, obtained under identical conditions with rat-heart sarcosomes. The activity of the blowfly sarcosomes can be increased by adding either bovine plasma albumin or 2:4-dinitrophenol (Slater & Lewis, 1953), neither of which has any effect on the rat-heart sarcosomes.

The experiments with albumin were prompted by the paper of Watanabe & Williams (1953) which showed that a solution of 2·5% bovine plasma albumin in 0·16M potassium phosphate buffer, pH 7·0, was the optimum medium for the preservation of the microscopic appearance of isolated sarcosomes. Although we did not find this medium as suitable as sucrose for isolating sarcosomes with a high P:O ratio, we did find that the addition of albumin to the reaction mixture had a remarkable effect on the oxidation of α-ketoglutarate. The full effect was usually obtained with the lowest concentration tested (0·12%), which was considerably below the concentration in the isolation medium of Watanabe & Williams. The oxygen uptake proceeded at practically a uniform rate (in the presence of ethylenediaminetetraacetic acid) whether or not albumin was added. This shows clearly that albumin acts not just by stabilizing the system, but as a true activator.

Sacktor (1954) has also reported that albumin increased the rate of oxygen uptake with housefly sarcosomes oxidizing α-ketoglutarate. However, the chief effect of albumin in his experiments was on the phosphorylation. No uptake of phosphate occurred in the absence of albumin, while 2% or more of albumin was necessary for maximum phosphorylation. In our experiments, because ethylenediaminetetraacetic acid was in the reaction mixture, albumin had only a slight effect (or none) on the phosphorylation ratios with α-ketoglutarate as substrate. However, it considerably increased the P:O ratio when succinate was the substrate. For this reason and because of the marked stimulation of the rate of oxygen uptake, we would agree with Watanabe & Williams (1953) and Sacktor (1954) that it is advantageous to include albumin in the reaction mixture.

The mechanism of the activation by albumin is obscure. It is unlikely to be the same as that suggested for 2:4-dinitrophenol (Slater & Lewis, 1953) for two reasons: (1) the α-ketoglutaric oxidase systems of some preparations were only slightly stimulated by 2:4-dinitrophenol, but markedly accelerated by albumin; (2) albumin did not decrease the phosphorylation ratios like 2:4-dinitrophenol.

Some other treatments which caused decreased phosphorylation ratios also increased the activity of the α-ketoglutaric oxidase system, for example isolation with hypotonic sucrose or with isotonic saline. The explanation in these cases might be the same as that given for 2:4-dinitrophenol. In any case, these results show that the activity of the α-ketoglutaric oxidase system is by itself of little value in assessing the effect of a particular treatment of the blowfly sarcosomes. This is different from the behaviour of rat-heart sarcosomes in which case the activity of the α-ketoglutaric oxidase system is a more sensitive index than the phosphorylation ratios (Slater & Cleland, 1953*a*).

Effect of ethylenediaminetetraacetic acid

Although the effect of adding ethylenediaminetetraacetic acid to the reaction mixture was less striking than in similar experiments with rat-heart sarcosomes (Slater & Cleland, 1953*b*), it was still quite appreciable, in the absence of albumin. A high concentration of albumin appears to be able to substitute for the chelating agent, as well as having a marked effect on its own, since the addition of ethylenediaminetetraacetic acid to a medium already containing 0·37% albumin had little effect on either the rate of oxidation of α-ketoglutarate or on the phosphorylation ratios. This may be because albumin, in the high concentrations used, can also effectively bind calcium (Greenberg, 1944; Katz & Klotz, 1953). Our results are in complete opposition to the surprising finding of Sacktor (quoted by Watanabe & Williams, 1953) that the addition of 0·001M ethylenediaminetetraacetic acid abolished the oxidation of α-ketoglutarate by isolated insect sarcosomes. Sacktor (1954) does not refer to this experiment, but states that the inclusion of 0·01M ethylenediaminetetraacetic acid in the isolation medium (0·25M sucrose) completely inhibited both respiration and phosphate uptake. We are also in complete disagreement with this result (see Table 3).

The inclusion of ethylenediaminetetraacetic acid in the isolation medium had no appreciable effect on the phosphorylative activity of sarcosomes tested immediately after preparation in a reaction mixture containing the chelating agent. However, such preparations, like the corresponding preparations from heart, could be incubated at 25° for 15 min. without inactivation of oxidative phosphorylation, in contrast to the considerable inactivation in sarcosomes isolated in sucrose alone.

P:O *ratio*

It is always a difficult problem to assess the activity of enzymes *in vivo* from studies with cell-free preparations. In the case of the P:O ratio of

rat-heart sarcosomes, we were unusually well-placed, since the ratio was found to be almost independent of considerable variations in the isolation medium, the reaction mixture and the treatment of the sarcosomes (Slater & Holton, 1954). It was possible then to extrapolate from the situation in the isolated sarcosome to that in the intact cell with some degree of confidence.

The blowfly sarcosomes present a more difficult problem. The P:O ratio was found to depend in a critical manner on the isolation procedure, which in comparison with that used for isolating heart sarcosomes was very mild, and on variations of the composition of the reaction mixture. Moreover, there were large unexplained variations in the P:O ratio from experiment to experiment, using apparently the same procedure with blowflies bred in the same way.

There are three possible explanations for the finding that the phosphorylation ratios obtained with blowfly sarcosomes are considerably below those obtained with rat-heart sarcosomes under identical conditions. (1) The yield of phosphorylation in blowfly sarcosomes, *in vivo*, may be lower than in mammalian heart. (2) The phosphorylation enzymes of the blowfly sarcosomes may be more labile than the corresponding enzymes in the rat-heart sarcosomes. (3) The P:O ratio of blowfly and rat-heart sarcosomes are identical *in vivo*, there is no difference in the stability of the respective enzymes, but the activity of the phosphorylating enzymes is not the rate-limiting step in the rat-heart sarcosome, but is limiting in the blowfly sarcosome. Thus the same degree of inactivation of a phosphorylating enzyme could conceivably have no effect on the P:O ratio in the rat-heart sarcosomes, but appreciably depress the ratio in the blowfly sarcosome.

The available evidence does not allow us to choose between these three alternatives.

SUMMARY

1. Oxidative phosphorylation has been demonstrated with sarcosomes isolated from the thoracic muscle of the blowfly *Calliphora erythrocephala*.

2. The phosphorus:oxygen ratio varied considerably from preparation to preparation, but never exceeded 2·0. The ratio was lower with sarcosomes isolated from young flies.

3. Factors causing low ratios were (i) isolation in media hypotonic or hypertonic to the blowfly haemolymph (tonicity equal to that of 0·36 M sucrose); (ii) isolation in potassium chloride or phosphate; (iii) allowing the dissected thoraces to stand at room temperature for 35 min.; (iv) subjecting the thoraces to more than gentle crushing to liberate the sarcosomes; (v) omission of ethylenediaminetetraacetic acid from the reaction mixture; (vi) prolonging the experiment beyond 35 min. (at 25°). The treatments referred to in (i) and (ii) gave preparations with higher oxidative activity.

4. The inclusion of fluoride or malonate in the reaction mixture or variation of the α-ketoglutarate concentration between 0·002 and 0·01 M had no effect on the phosphorylation ratios.

5. Sarcosomes isolated in sucrose containing ethylenediaminetetraacetic acid oxidized α-ketoglutarate more slowly than those isolated in sucrose alone. The P:O ratio was little affected.

6. The addition of small amounts of albumin (1·2 mg./ml.) to the reaction mixture markedly stimulated the rate of oxidation of α-ketoglutarate, without usually affecting the phosphorylation ratio. Albumin considerably increased the P:O ratio when succinate was the substrate.

7. Albumin is a true activator of the oxidase system, in contrast with ethylenediaminetetraacetic acid which stabilizes the system.

8. Apart from the activation caused by the introduction of albumin into the reaction mixture, the inclusion of albumin in the isolation medium had little effect.

This work was carried out while S. E. L. was on leave from D.S.I.R. Pest Infestation Laboratory, London Road, Slough. E.C.S. is working on behalf of the Agricultural Research Council. We wish to thank Professor D. Keilin, F.R.S., and Dr P. Tate for their interest and advice.

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APPENDIX

Relationship Between Amount of Hexokinase and Amount of Phosphate Esterified

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(*Received* 13 *April* 1954)

The reactions concerned may be expressed in a simplified form as follows

$$\xrightarrow{k_1} \text{ATP}, \quad (1)$$

$$\text{ATP} + \text{glucose} \xrightarrow{k_2[\text{hK}]} \text{HMP} + \text{ADP}, \quad (2)$$

$$\text{ATP} + \text{H}_2\text{O} \xrightarrow{k_3} \text{ADP} + \text{H}_3\text{PO}_4. \quad (3)$$

Reaction (1) represents the rate of synthesis of ATP by the oxidative phosphorylation reaction. Except at very low concentrations of ADP, this is independent of the concentration of ADP (Slater & Holton, 1953). Reaction (2) is the hexokinase reaction; it is assumed that the concentration of ATP is low, so that with saturating concentrations of glucose, the rate of this reaction will be proportional to the concentrations of ATP and to the concentration of hexokinase (hK). Reaction (3) is the ATPase reaction; again it is assumed that the ATP concentration is so low that the rate of reaction (3) is proportional to it. Since the enzymes catalysing reactions (1) and (3) are in the sarcosomal preparation, the amount of which is not varied, the concentrations of these enzymes are included in the rate constants k_1 and k_3.

In the steady state,

$$\text{d[ATP]}/\text{d}t = k_1 - [\text{ATP}]\,(k_2[\text{hK}] + k_3) = 0, \quad (4)$$

$$\text{d[HMP]}/\text{d}t = v = k_2[\text{hK}]\,[\text{ATP}]. \quad (5)$$

Adding (4) and (5),

$$v = k_1 - k_3[\text{ATP}]. \quad (6)$$

From (4),

$$[\text{ATP}] = \frac{k_1}{k_2[\text{hK}] + k_3}. \quad (7)$$

Combining (6) and (7)

$$v = k_1\left[1 - \frac{k_3}{k_3 + k_2[\text{hK}]}\right],$$

i.e.

$$v = \frac{k_1 k_2[\text{hK}]}{k_3 + k_2[\text{hK}]},$$

and

$$1/v = \frac{k_3 + k_2[\text{hK}]}{k_1 k_2[\text{hK}]}.$$

Thus

$$\frac{1}{v} = \frac{1}{k_1} + \frac{k_3}{k_1 k_2}\cdot\frac{1}{[\text{hK}]}. \quad (8)$$

That is, $1/v$ is a linear function of $1/[\text{hK}]$.

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The *in vitro* Enzymic Hydroxylation of Steroid Hormones

2. ENZYMIC 11β-HYDROXYLATION OF PROGESTERONE BY OX-ADRENOCORTICAL MITOCHONDRIA

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(*Received* 20 *April* 1954)

On the basis of experiments in which steroids were perfused through ox-adrenal glands, Hechter *et al.* (1951) were able to present a tentative scheme of adrenocortical hormone biogenesis. Progesterone is postulated as a key intermediate in the scheme, since perfusion of this steroid gave the following products: 17α-hydroxyprogesterone (3–7%), 17α-hydroxycorticosterone (10–12%), corticosterone (1·2–2·4%) and 11β-hydroxyprogesterone (less than 1%). The figures in parenthesis indicate the amounts of steroid recovered. Using ox-adrenal 'homogenates' prepared in potassium chloride solution Hayano & Dorfman (1952) confirmed the ability of the gland to form corticosterone from progesterone. In subsequent experiments using 'residue preparations of twice washed ox-adrenal "homogenates" obtained at 5000 *g*' the same workers (Hayano & Dorfman, 1953) failed to convert progesterone into 11β-hydroxyprogesterone under conditions which permitted the 11β-hydroxylation of 11-deoxycorticosterone (DOC) to take place. They attributed this failure to the absence of a hydroxyl group at the 21-position in the progesterone molecule. The 21-hydroxylating enzyme which Plager & Samuels (1953) have shown to be present in the 'supernatant fraction' of a 'homogenate' centrifuged at 20000 *g* would presumably have been removed on washing the sedimented particles used by Hayano & Dorfman.

In a previous paper (Brownie & Grant, 1954*a*) it was shown that the 11β-hydroxylation of DOC by ox-adrenocortical mitochondria requires concurrent oxidative phosphorylation, for which citric acid cycle intermediates are oxidizable substrates. The observation of Grant & Taylor (1952) that progesterone has a greater inhibitory action than DOC on certain reactions of the tricarboxylic acid cycle in rat-liver mitochondria suggested that similar effects in adrenocortical mitochondria might explain the failure of Hayano & Dorfman (1953) to obtain 11β-hydroxylation of progesterone. Consequently it was decided to study conditions for the 11β-hydroxylation of progesterone by ox-adrenocortical mitochondria with particular reference to the influence of progesterone on reactions contributing to or supporting the hydroxylation reaction. The results of this investigation are now reported.

A preliminary account of this work has been communicated to the Biochemical Society (Brownie & Grant, 1954*b*).

EXPERIMENTAL

Materials and Methods

Melting points were determined on a hot-stage type of apparatus and are corrected. Progesterone was purified by recrystallization from *n*-hexane and aqueous ethanol to give a product melting at 121–121·5°. DOC was prepared from the acetate as described by Brownie & Grant (1954*a*). 11β-Hydroxyprogesterone was a crystalline specimen, m.p. 187–189°. Reichstein & Fuchs (1940) reported m.p. 187–188° for this steroid.

A commercial preparation of the sodium salt of adenosine triphosphate (ATP) (L. Light and Co. Ltd., Slough) was used. Alumina (Peter Spence and Sons Ltd., Widnes) activity II (Brockmann & Schodder, 1941) was used for adsorption chromatography.

In the isolation of ox-adrenocortical mitochondria as described by Brownie & Grant (1954*a*), it has now been found more convenient to use a small Latapie mincer in the cold room at 0° for the preliminary disintegration of the tissue.

Celite 545 and solvents were purified as described by Taylor (1954).

For quantitative experiments, citric acid cycle intermediates and suspensions of mitochondria in the concentrations stated were added to a basal reaction mixture containing 0·095 M-KCl, 0·004 M-$MgSO_4$, 0·0005 M potassium ATP and 0·04 M potassium phosphate (pH 7·4) in a total vol. of 3 ml. Steroids were added in solution in propylene glycol (propane-1:2-diol). Unless otherwise stated, incubations were in air, shaking for 1 hr. at 37°. Each experiment was conducted in four parts as already described (Brownie & Grant, 1954*a*). Incubations were terminated by addition of 15 ml. cold acetone, except when citrate was to be determined, in which case 3 ml. 30% (w/v) trichloroacetic acid was added. After centrifuging, citrate was determined in a portion of the supernatant solution by the method of Taylor (1953).

Oxygen consumption measurements were made by the usual Warburg technique (Umbreit, 1949).

Determination of DOC, progesterone and 11β-hydroxyprogesterone

Steroids were determined by the method described by Taylor (1954) for progesterone with slight modifications. Acetone extracts were not chilled before filtering and benzene–$CHCl_3$ (6:1, v/v) was used for the extraction of

aqueous-acetone residues. The modifications introduced by Brownie & Grant (1954*a*) were used in the method for DOC. Progesterone and 11β-hydroxyprogesterone present together in incubated mixtures were determined in separate portions of the benzene–$CHCl_3$ extract. 11β-Hydroxyprogesterone was isolated by partition chromatography on a Celite column (5 g. Celite 545/4 ml. methanol–water (7:3, v/v)); the solvent systems were benzene–*n*-hexane (3:7, v/v), and methanol–water (7:3, v/v). 11β-Hydroxyprogesterone was eluted in the fraction from 20 to 40 ml. DOC if present would be eluted in the same fraction, but this steroid was not found among the products obtained on incubating washed adrenocortical mitochondria with progesterone.

Steroids eluted from chromatogram columns were determined by measuring their selective absorption at 240 mμ. in ethanol in a Unicam SP 500 spectrophotometer. Amounts of steroid present were found by reference to calibration curves.

Typical results for the recovery of progesterone and 11β-hydroxyprogesterone added to the same incubation mixture containing 200 mg. wet weight mitochondria are shown in Table 1.

'Apparent' steroid recovered in experiments in which no steroids were used was consistently very low (approx. 20 μg.). These values were subtracted in all steroid determinations.

RESULTS

Influence of progesterone and DOC on reactions of the tricarboxylic acid cycle supporting 11β-hydroxylation

The influence of progesterone and DOC on citrate metabolism by adrenocortical mitochondria was investigated by measuring the amount of citrate accumulating in reaction mixtures on incubation with pyruvate and L-malate. From the results of a typical experiment shown in Fig. 1 (*a*) it is seen that much less citrate accumulates in the presence of progesterone than with an equivalent amount of DOC. This might be due to a greater inhibition by progesterone of citrate-synthesizing reactions or a greater stimulation of citrate oxidation by progesterone. Direct measurement of the effect of progesterone and DOC on citrate oxidation showed that both steroids inhibited this reaction to about the same extent (Fig. 2). It would thus appear that progesterone exerts a more powerful inhibitory effect on the synthesis of citrate from pyruvate and L-malate than does DOC. The inhibitory action of DOC on citrate synthesis is eliminated and that of progesterone is somewhat decreased if increased amounts of enzyme are used (Fig. 1, *b*, *c*).

Another example of the greater inhibition by progesterone than by DOC is given in Fig. 3 (*a*), which shows the effect of these steroids on the oxygen consumption of mitochondria incubated with succinate.

Concurrent with these measurements of oxygen consumption, rates of metabolism of progesterone and DOC were determined. The results shown in Fig. 3 (*b*) indicate that the added DOC is almost completely metabolized in the first 10 min. of

Table 1. *Recovery of progesterone and 11β-hydroxyprogesterone added to reaction mixtures which had been incubated with adrenocortical mitochondria*

Values have been corrected by subtraction of 'apparent' steroid recovered in blank experiments in which no steroids were added.

Added (μmoles)	Recovered (μmoles)	Recovery (%)
	Progesterone	
1·83	1·75	96
1·65	1·56	95
0·744	0·716	96
0·744	0·716	96
	11β-Hydroxyprogesterone	
1·23	1·18	96
1·23	1·17	95
0·712	0·636	89
0·712	0·621	87

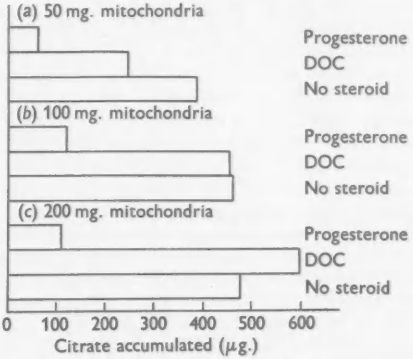

Fig. 1. Effect of progesterone and DOC on citrate accumulation by ox-adrenocortical mitochondria incubated in air for 1 hr. at 37°. Basal reaction mixture supplemented with 0·016 M potassium pyruvate, 0·016 M potassium L-malate and wet weights of mitochondria shown. 500 μg. steroid added in 0·04 ml. propylene glycol.

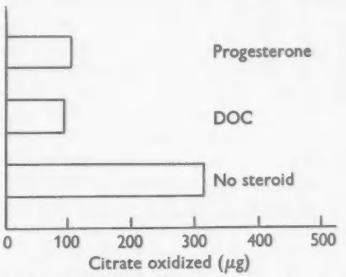

Fig. 2. Effect of progesterone and DOC on citrate oxidation by ox-adrenocortical mitochondria incubated in air for 1 hr. at 37°. Basal reaction mixture supplemented with 0·001 M sodium citrate (576 μg. citric acid/flask) and 100 mg. wet weight mitochondria. 500 μg. steroid added in 0·04 ml. propylene glycol.

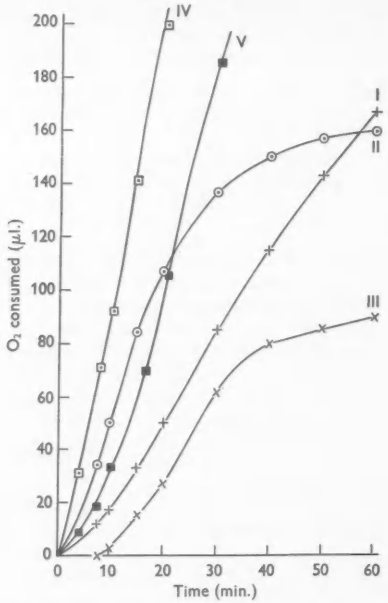

Fig. 3 (*a*). Effect of progesterone and DOC on succinate oxidation by ox-adrenocortical mitochondria. All flasks contained basal reaction mixture. 500 μg. steroids were added in 0·04 ml. propylene glycol. I. 100 mg. wet weight mitochondria, 0·002 M succinate. II. As I plus DOC. III. As I plus progesterone. IV. 200 mg. wet weight mitochondria, 0·01 M succinate. V. As IV plus progesterone.

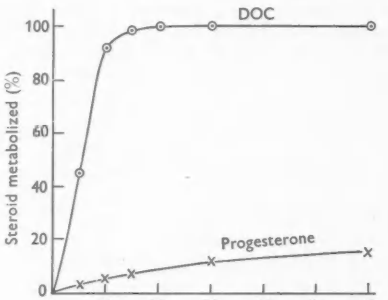

Fig. 3. (*b*). Rates of metabolism of progesterone and DOC by ox-adrenocortical mitochondria. Basal reaction mixture was supplemented with 0·02 M succinate and 100 mg. wet weight mitochondria. 500 μg. steroids were added in 0·04 ml. propylene glycol. Incubations in air at 37°.

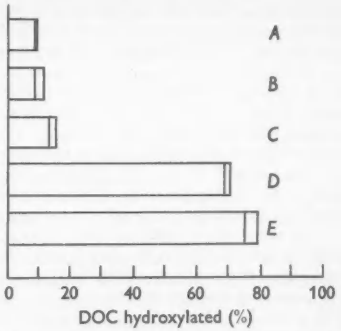

Fig. 4. Effect of pre-incubation of ox-adrenocortical mitochondria on DOC metabolism. 100 mg. wet weight mitochondria were added to the basal reaction mixture. 500 μg. of steroids were added in 0·04 ml. propylene glycol. Incubations were in air at 37°. (Double lines at ends of bars indicate duplicate values.) *A*. Progesterone, DOC and succinate (0·002 M) present from the start, 10 min. incubation. *B*. Progesterone and succinate (0·002 M) present from the start. After 20 min. pre-incubation DOC was added and incubation continued for further 10 min. *C*. After 20 min. pre-incubation, DOC and succinate (0·002 M) were added and incubation continued for further 10 min. *D*. Succinate (0·002 M) present from the start. After 20 min. pre-incubation DOC was added and incubation continued for further 10 min. *E*. DOC and succinate (0·002 M) present from the start, 10 min. incubation.

incubation, a period during which there is a marked stimulation of oxygen consumption. In contrast with this, added progesterone inhibits oxygen consumption and is not itself appreciably metabolized during the first 10 min. This suggests that the adrenocortical mitochondrial enzymes directly or indirectly involved in steroid 11β-hydroxylation may be 'inactivated' during the period in which the mitochondria are not actively respiring. In order to investigate this possibility, mitochondria were pre-incubated under the conditions shown in Fig. 4. The ability of the enzyme preparation to catalyse the 11β-hydroxylation of DOC was then measured. From the results shown in Fig. 4, it is clear that pre-incubation without oxidizable substrate (succinate) or with succinate oxidation inhibited by progesterone, greatly reduces the 11β-hydroxylation of DOC added subsequently. This loss of 11β-hydroxylating activity is not observed if succinate oxidation is uninhibited during the pre-incubation period (bar *D* of Fig. 4).

Determination of conditions suitable for metabolism of progesterone by ox-adrenocortical mitochondria

The results of experiments described above suggested that the inhibition of reactions supporting the 11β-hydroxylation of DOC in ox-adrenocortical

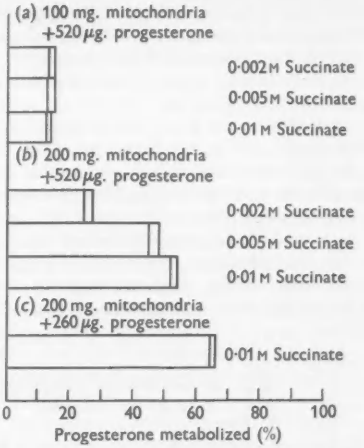

Fig. 5. Effect of varying concentrations of succinate, mitochondria and steroid on progesterone metabolism by ox-adrenocortical mitochondria. Basal reaction mixture supplemented with concentrations of succinate and mitochondria shown. Progesterone in propylene glycol added in the amounts shown. Incubation in air for 1 hr. at 37°. Double lines at ends of bars indicate duplicate values.

mitochondria by progesterone might account for the failure to hydroxylate progesterone itself under these conditions. Conditions were therefore sought which would decrease the inhibitory action of progesterone on the reactions supporting steroid 11β-hydroxylation.

From the results of experiments in which progesterone, succinate and enzyme concentrations were varied (Fig. 5) it appears that incubation of approximately 500 μg. progesterone with 0·01 M succinate and 200 mg. wet weight packed mitochondria (about 6 mg. total N) results in about 50 % metabolism of the progesterone. When the amount of progesterone is reduced to approximately 250 μg. about 65 % is metabolized.

Investigation of the products of metabolism of progesterone by ox-adrenocortical mitochondria

Consideration of the results described in the previous section indicated that the use of 0·01 M succinate and a progesterone:wet packed mitochondria weight ratio of 1:800 in incubation mixtures might be expected to result in 65 % metabolism of the steroid. The difficulty in preparing large amounts of mitochondria from the cortical tissue of fresh ox adrenals limited the amount of steroid which could be incubated at any one time. It appeared that incubation of 45 mg. of progesterone would be convenient for the isolation of the main products of metabolism. Consequently, separate 5 mg. samples of progesterone in 0·4 ml. propylene glycol were incubated on each of 9 days with 4 g. wet packed mitochondria in 60 ml. of the basal reaction mixture containing 0·01 M succinate. This volume was distributed in a number of conical flasks which were shaken in air for 1 hr. at 37°. Progesterone determinations on small portions of the mixture after incubation indicated that about 60 % of the steroid had been metabolized. A part of the residue from the benzene–$CHCl_3$ extract prepared for the progesterone determinations was chromatographed on a Celite column using the solvent system benzene–hexane (3:7, v/v), methanol–water (7:3, v/v) normally used in DOC determinations. Observation of the elution of material absorbing selectively at 240 mμ. indicated the presence of a substance slightly more polar than DOC. On oxidation with periodate this substance yielded no formaldehyde as determined by the method of Daughaday, Jaffe & Williams (1948). This indicated that it was not a steroid having an α-ketol side chain in the 17-position. The observed chromatographic behaviour of this substance determined the procedures employed for its subsequent isolation and purification.

Incubations were terminated by the addition of 5 vol. of chilled acetone and precipitated material was filtered off after standing 2 hr. at −20°. The precipitate was washed with cold acetone. The filtrate and washings were evaporated *in vacuo* to an aqueous residue of about 50 ml. This was diluted with an equal volume of water, saturated with NaCl, and extracted with 3 × 50 ml. ethyl acetate.

The pooled ethyl acetate extracts were washed with 50 ml. 0·2 N-$NaHCO_3$, 50 ml. 0·2 N-HCl and finally with water until the washings were neutral, dried (Na_2SO_4) and evaporated to dryness *in vacuo*. The residue obtained was partitioned in 100 ml. of a solvent system consisting of 20 ml. *n*-hexane and 80 ml. methanol–water (80:20, v/v). Much yellow material entered the less polar hexane phase. The methanol–water phase was evaporated *in vacuo* to an aqueous residue, which was diluted to 50 ml. with water and extracted with 3 × 50 ml. $CHCl_3$. The pooled $CHCl_3$ extract was dried (Na_2SO_4) and evaporated to dryness *in vacuo*.

At this stage the residues from extracts of individual incubation mixtures were combined and submitted to a seven-stage countercurrent distribution with double withdrawal (Craig & Craig, 1950). This was carried out in separating funnels with the solvent system methanol–water (80:20, v/v), benzene–*n*-hexane (80:20, v/v), using 50 ml. of each phase. The partition coefficient of progesterone in this system was 0·23 and that of 11β-hydroxyprogesterone 1·6. Fig. 6 shows the theoretical distribution of these two steroids in such an experiment and also the actual distribution of isolated

material absorbing selectively at 240 mμ. Residues obtained on evaporating the contents of funnels 0–6 to dryness *in vacuo* were semi-crystalline. Their combined weight was 12·5 mg. A solution of these combined residues in 3 ml. benzene–*n*-hexane (1:1, v/v) was poured on to a column of about 4 mm. diameter containing 400 mg. Al_2O_3. The chromatogram was developed as shown in Table 2.

The white crystalline material from fractions 7 to 18 was recrystallized from benzene–*n*-hexane (1:1, v/v) to give 8 mg. of crystals melting at 189–191°. On mixing with an authentic specimen of 11β-hydroxyprogesterone, m.p. 187–189°, the m.p. was 187–189°.

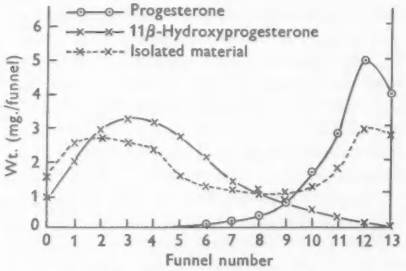

Fig. 6. Theoretical distribution of progesterone and 11β-hydroxyprogesterone and the distribution of isolated material absorbing selectively at 240 mμ. in a seven-stage countercurrent distribution with double withdrawal using the solvent system benzene–*n*-hexane (80:20, v/v) and methanol–water (80:20, v/v). Volume of each phase was 50 ml.

The isolated material and 11β-hydroxyprogesterone (70 μg. each) in stoppered tubes were separately treated with 5 ml. conc. H_2SO_4 for 2 hr. at 17°. Both solutions gave identical absorption spectra over the range 220–520 mμ. (Fig. 7).

The infrared spectra of the isolated material melting at 181–191° and of authentic 11β-hydroxyprogesterone were determined with mulls ground in paraffin in a double-beam Perkin-Elmer 21B spectrophotometer. The positions of all the bands and their relative intensity patterns within each spectrum were identical. From the evidence thus obtained there can be no doubt that the isolated material is 11β-hydroxyprogesterone.

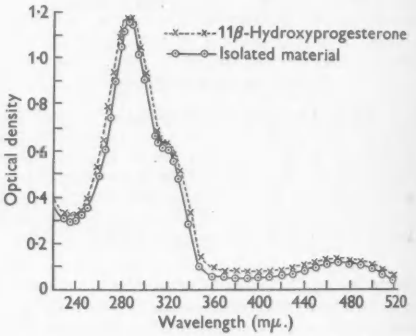

Fig. 7. Absorption spectra of solutions of 11β-hydroxyprogesterone and of isolated crystalline material in concentrated H_2SO_4.

Table 2. *Chromatography on Al_2O_3 of residues from funnels 0–6 in the countercurrent distribution of material obtained on incubation of progesterone with ox-adrenocortical mitochondria*

Each fraction, 3 ml. Solvent ratios, all v/v.

Fraction no.	Solvent	Residue	
		Wt. (mg.)	Description
1–3	Benzene–*n*-hexane (1:1)	—	—
4–6	Benzene–*n*-hexane (3:2)	—	—
7–9	Benzene–*n*-hexane (4:1)	0·5	White crystals
10–12	Benzene	1·4	White crystals
13–15	Benzene–ether (9:1)	6·0	White crystals
16–18	Benzene–ether (4:1)	0·5	White crystals
19–21	Benzene–ether (3:2)	0·5	Amorphous solid

Table 3. *Recovery of progesterone and 11β-hydroxyprogesterone following incubation of progesterone with ox-adrenocortical mitochondria*

Figures in parenthesis indicate the number of results obtained.

Expt.	Progesterone added (μmoles)	Progesterone recovered (μmoles)	11β-Hydroxyprogesterone recovered (μmole)	Total recovery	
				(μmoles)	(%)
1	1·60	1·1±0·05 (8)	0·37±0·04 (8)	1·47±0·1	92±6
2	1·54	0·9±0·06 (8)	0·42±0·07 (8)	1·32±0·13	85±8

The results of quantitative experiments, shown in Table 3, indicate that the 11β-hydroxyprogesterone found may account for the greater part of the progesterone metabolized by ox-adrenocortical mitochondria. Attempts were made to obtain information on the nature of other metabolic products by paper chromatography of the benzene–$CHCl_3$ extract obtained in quantitative experiments. Portions of extract containing about 150 μg. material absorbing selectively at 240 mμ. were employed. For enzyme blanks, similar portions were taken from extracts obtained after incubation of mitochondria without added steroids. For standards, about 150 μg. each of progesterone, 11β-hydroxyprogesterone and DOC were added to separate portions of blank extract. The chromatograms were run in duplicate simultaneously for 2 hr. at 18° using the solvent system B_1 of Bush (1952). Dried papers were sprayed with (*a*) methanolic-NaOH reagent (Bush, 1952) which detects steroids having an αβ-unsaturated 3-ketone structure in concentrations of 2 μg./cm.² and (*b*) blue tetrazolium reagent (Mader & Buck, 1952) which detects steroids having an α-ketol side chain in concentrations of 1–2 μg./cm.². Spots corresponding in position to 11β-hydroxyprogesterone and progesterone were observed on chromatograms of extracts from progesterone incubation experiments. No trace of DOC was observed on these chromatograms, but the blue tetrazolium reagent showed the presence of a trace of material more polar than DOC. This was not observed on chromatograms of progesterone or with the mitochondrial blanks.

DISCUSSION

Hechter (1953*a*) cautiously describes the scheme of adrenocortical-steroid biogenesis which he previously advanced (Hechter *et al.* 1951) as 'a series of deductions based primarily upon the ability of substances to react', and admits the possibility that such *in vitro* reactions may be relatively unimportant in normal metabolic pathways. When evaluating the physiological significance of *in vitro* reactions of adrenocortical steroids and their possible precursors, it is important to bear certain points in mind. The state of enzymes employed and the influence of steroids on enzyme reactions are factors which appear to the present authors to merit special attention.

The unsatisfactory nature of the 'homogenates' and 'washed sediments' used as sources of enzymes for the study of adrenocortical steroid metabolism has already been discussed (Brownie & Grant, 1954*a*). The skilful adaptation of the perfusion technique by Hechter *et al.* (1953) might be expected to preserve adrenocortical enzymes in a natural state, but the results obtained may have been influenced by exposing the perfused steroids to deliberately damaged adrenal tissue.

It is well known that mitochondria, carefully isolated under suitable conditions, contain enzymes and coenzymes which catalyse a wide variety of important reactions including the transfer of hydrogen and electrons to molecular oxygen. In particular, Kielley & Kielley (1951) have shown that such mitochondria catalyse high rates of phosphorylation coupled with oxidation of metabolites of the citric acid cycle. This sequence of reactions has been shown to be necessary for the 11β-hydroxylation of DOC by adrenocortical mitochondria (Brownie & Grant, 1954*a*).

While it can be argued that isolated mitochondria may be rapidly altered from their natural state by incubation in media which inadequately represent the intracellular fluid, it is notable that only very brief incubations at 37° are necessary in order to obtain extensive 11β-hydroxylation of steroids. These observations encourage the belief that carefully prepared mitochondria are suitable sources of enzymes for the study of certain reactions of adrenocortical-steroid biogenesis *in vitro*.

Little attention appears to have been paid to the influence of other steroids on enzyme reactions involved in the biogenesis of adrenocortical steroids. Hechter (1953*b*) has referred briefly to the possible influence of sex hormones on the pattern of adrenocortical steroids synthesized and released from the gland.

Sourkes & Heneage (1952) have observed a stimulation by DOC of succinate oxidation by rat-adrenal halves. Brummel, Halkerston & Reiss (1954) have made the same observation with ox-adrenal slices. This stimulation has now been observed using ox-adrenocortical mitochondria. At the same time the DOC is extensively converted into corticosterone. The oxygen required for this reaction does not however account for the increased oxygen consumption observed.

In contrast with the effect of DOC it has now been found that progesterone inhibits succinate oxidation by adrenocortical mitochondria, and that under such circumstances the 11β-hydroxylation of the steroid is inhibited. It has also been observed in the present work that progesterone has a greater inhibitory action than DOC on reactions involved in the synthesis of citrate from pyruvate and L-malate. It is possible that the inhibitory action of progesterone restricts 'high-energy' phosphate production with resultant inhibition of 11β-hydroxylation of the steroid.

The inhibition by progesterone is most marked during the first 10 min. of incubation. Thereafter oxygen consumption is almost parallel with that in control experiments without added steroid (Fig. 3*a*). No explanation can be offered for this observation.

It appears, however, that enzymes indirectly or directly concerned with 11β-hydroxylation of steroids by adrenocortical mitochondria suffer permanent damage during the first 10 min. of incubation if tricarboxylic acid cycle intermediates are absent, or if their oxidation is inhibited (Fig. 4). This may be explained by the well-known instability under these conditions of enzymes involved in oxidative phosphorylation.

The limited increase in steroid hydroxylation observed on increasing the concentration of enzyme in the case of progesterone as compared with DOC (Fig. 5) suggests that the progesterone effect may not be restricted to an inhibition of respiration. The increased rate of ATP breakdown by rat tissues in the presence of progesterone observed by Jones & Wade (1953) offers an additional explanation for the influence of progesterone on reactions concerned with 11β-hydroxylation.

By suitable adjustment of the concentrations of progesterone, succinate and mitochondria it has now been found possible to decrease the effect of the progesterone on reactions of the tricarboxylic acid cycle sufficiently to permit appreciable 11β-hydroxylation of this steroid.

In the scheme of adrenocortical-steroid biogenesis advanced by Hechter *et al.* (1951), 11β-hydroxyprogesterone is shown as a possible intermediary in the formation of corticosterone or 17α-hydroxycorticosterone from progesterone. The status of 11β-hydroxyprogesterone as such as intermediary has been diminished by the following facts. This steroid has hitherto only been isolated in yields of less than 1 % after perfusion of progesterone through adrenal glands. It has not been found among the products obtained on incubation of adrenocortical tissue preparations with progesterone (Hayano & Dorfman, 1952, 1953). Finally, 11β-hydroxyprogesterone has not been converted into 17α-hydroxycorticosterone or corticosterone in significant amounts (Hechter, 1953*a*). Since it has now been shown to be possible to convert progesterone into 11β-hydroxyprogesterone in good yield by adrenocortical mitochondria it would appear that the status of the latter steroid as an intermediary in the biogenesis of adrenocortical steroids should be reconsidered. The conditions under which attempts have been made to convert 11β-hydroxyprogesterone into corticosterone or 17α-hydroxycorticosterone have not been described by Hechter (1953*a*). It is therefore not at present possible to decide whether these reactions may or may not occur.

The elucidation of the mechanism of adrenocortical-steroid biogenesis is further limited by lack of knowledge of the influence of steroids other than progesterone on the metabolism of their precursors and of the relative affinities of the enzymes concerned for the various steroid substrates which may be present. These problems must form the subjects for future investigations.

SUMMARY

1. Progesterone has been found to be a more powerful inhibitor than 11-deoxycorticosterone (DOC) of certain reactions of the tricarboxylic acid cycle in ox-adrenocortical mitochondria.

2. These inhibitions offer some explanation of the failure of steroid 11β-hydroxylation in presence of progesterone, since this reaction in adrenocortical mitochondria appears to depend upon oxidative phosphorylation for which members of the tricarboxylic acid cycle are oxidizable substrates.

3. Conditions are described under which progesterone may be converted by ox-adrenocortical mitochondria into 11β-hydroxyprogesterone as the main product, and to traces of a more polar product which reduces blue tetrazolium.

4. The choice of suitable enzyme preparations and the influence of steroids on enzymes are discussed in relation to the physiological significance of steroid reactions observed *in vitro*.

It is a pleasure to record our appreciation of the interest which Professor G. F. Marrian, F.R.S., has shown in this work, and to thank him for his encouragement. The expenses of the work were defrayed in part by a grant to Professor Marrian from the Medical Research Council. The DOC acetate was a generous gift from Dr C. L. Hewett, Organon Laboratories Ltd., Newhouse, Lanarkshire. The 11β-hydroxyprogesterone was kindly given by Dr R. Levine, The Upjohn Company, Kalamazoo, Mich., U.S.A. We are indebted to Dr L. J. Bellamy, Chemical Inspectorate, Ministry of Supply, London, for the determination of the infrared spectra. The co-operation of the manager and staff of the Offals Department, Wholesale Meat Marketing Association, Edinburgh, in the collection of fresh adrenal glands was of the greatest value. Mr R. Bayne purified the Celite and the solvents used.

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The Non-Protein Nitrogen of Extracts of *Pisum sativum*

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A number of methods have been used in the past for obtaining non-protein nitrogenous compounds from plant tissues, but little information about their relative efficiencies is available. The present study concerns the extraction of soluble nitrogen-containing compounds from green seed peas by cold and hot saline solutions and by cold and hot water. In these extracts the non-protein nitrogen (NPN) was determined after precipitation by trichloroacetic acid (TCA), ethanol, tungstic acid, colloidal iron and after dialysis. The NPN fractions were analysed for total N and carboxyl N (ninhydrin–carbon dioxide). The amino acids of the extracts were investigated by paper-partition chromatography.

MATERIAL AND METHODS

Green seed peas freshly removed from pod were used, the variety Onwards being studied. The green pods, collected approximately 20 days after flowering, were fully swollen and corresponded to stage 12 (Hyde, 1954).

Extraction. Approx. 10 g. of peas were ground in a glass mortar with 20 ml. of saline buffer (pH 7) of composition 0·2 M-NaCl, 0·3 M-Na_2HPO_4, 0·011 M-NaH_2PO_4. The supernatant liquid was decanted and the process repeated with seven successive lots of saline buffer to give a total volume of 140 ml. The extracts were centrifuged at 1500 *g* for 30 min. and the supernatant fluid was decanted. The extraction with cold distilled water was made in a similar manner. With the hot-saline and hot-water extractions, solutions at 98° were used.

Precipitation. (*a*) Trichloroacetic acid. To the pea extract (20 ml.) TCA (1 g.) was added, the mixture was allowed to stand for 45 min., the volume was measured and the mixture filtered.

(*b*) Ethanol. The pea extract (20 ml.) was mixed with ethanol (80 ml.) and the solution allowed to stand for 24 hr. at 2°. It was then filtered.

(*c*) Tungstic acid. To the pea extract (20 ml.) were added 10% (w/v) sodium tungstate (20 ml.) and 0·66 N-H_2SO_4 (20 ml.). The mixture was allowed to stand at 2° for 24 hr. and then filtered.

(*d*) Colloidal iron. This method was successful only when saline buffer was used as an extracting agent. Pea extract (10 ml.) was mixed with dialysed iron (10 ml.) (British Drug Houses Ltd.) and the solution left for 12 hr. at 2°. Water (40 ml.) was then added and the mixture shaken and filtered.

Dialysis. This was carried out by placing 50 ml. of the pea extract in a cellophan sac and dialysing against 5–6 vol. of chloroform water for 96 hr. at 0–2° with occasional stirring (Synge, 1951). The diffusate was concentrated *in vacuo* for further study.

Analytical methods. Nitrogen was determined by the micro-Kjeldahl method of Hiller, Plazin & Van Slyke (1948). The distillation apparatus of Markham (1942) was employed. In determinations of extractable N and of NPN, samples containing 0·24–0·47 mg. N were used. Determinations were in triplicate, and agreed within ±0·5%.

The ninhydrin–CO_2 method of Van Slyke, Dillon, MacFadyen & Hamilton (1941) with minor modifications was used. Pea extract (2 ml.) containing approx. 0·7 mg. carboxyl N was added to each tube. 2 ml. water were added in the case of the blank. It was found unnecessary to do blank determinations (CO_2 without ninhydrin) since these proved to be the same as water blanks. The determinations were made with trioxohydrindene hydrate (50 mg.) and 50 mg. citrate buffer (pH 2·5) using Thunberg vacuum tubes sealed with stopcock grease as reaction vessels. The various extracts were adjusted to a pH value suitable for the estimation. TCA was removed from the extract by means of ether, which raised the pH from 1 to 6. The ether dissolved in the extract caused an increase in pressure when the tube was evacuated but could be expelled after the CO_2 had been collected in the 0·5 N-NaOH. The ethanol extract was evaporated under reduced pressure at room temperature. The pH of the tungstic acid filtrate was adjusted to 2·5 by the addition of citrate buffer, excess CO_2 being removed by boiling the extract for 3 min. before the addition of

ninhydrin. No adjustment of pH was necessary for the extracts obtained after the use of colloidal iron or after dialysis. All determinations were carried out in triplicate; results agreed within $\pm 1°$.

To demonstrate the presence of amino acids in the extracts the two-dimensional paper-chromatographic method of Consden, Gordon & Martin (1944) was used. The solvent systems employed were phenol–ammonia (Dent, 1948) followed by *n*-butanol–acetic acid–water (4:1:5 by vol.) (Partridge, 1948).

Before chromatography was carried out, the extracts were treated as follows: (*a*) TCA when present was removed by repeated extraction with ether; (*b*) saline extracts were desalted (Consden, Gordon & Martin, 1947); (*c*) the treated solutions were concentrated *in vacuo* and the residues made up to a known volume with water. In each case the same volume was applied to the paper. The sodium tungstate extract was unsuitable for chromatography since the tungstate could not be removed. The amino acids were detected after drying by spraying with 0·1% (w/v) ninhydrin in ethanol.

RESULTS

The values for extractable N and for NPN in the various extracts following the use of the different treatments are given in Table 1. The results show that hot-saline extraction followed by TCA precipitation is the most effective method of extracting NPN from pea seeds. Extraction of NPN after ethanol precipitation is more effective when saline is used as the extracting agent, considerable adsorption of amino acids by protein taking place if water is used. Tungstic acid precipitation gives rise to low NPN values, since it appears that 'basic' nitrogen is precipitated by the acid. The colloidal-iron precipitation of protein is effective only if saline buffer is present. Dialysis, after extraction of the peas by either saline or water, gives similar values for NPN.

The results obtained in the determination of the carboxyl nitrogen in the extracts are given in Table 2. The values show that hot-saline extraction followed by TCA precipitation is an effective method of obtaining α-amino acids. Tungstic acid precipitation after hot-saline extraction gives the highest carboxyl N values. This may be due to precipitation and adsorption of the basic nitrogen by the protein thus leaving a higher percentage of α-amino acids in solution. With the other treatments hot-saline extracts have the higher carboxyl N values.

Chromatography. The following amino acids were recognized in all the extracts: aspartic acid, glutamic acid, serine, glycine, threonine, homoserine, alanine, α- and γ-aminobutyric acids, valine, methionine, leucine + isoleucine, proline, β-alanine, lysine, phenylalanine, citrulline, asparagine and glutamine. Three unidentified amino acids were present. Cysteic acid and pipecolinic acid were also probably present.

Differences in the extracts were as follows. With hot-saline extraction followed by TCA precipitation there was more glutamine, glutamic acid, serine, valine and leucine. No marked differences were obtained with hot- and cold-water and cold-saline extracts after TCA treatment.

The extracts after ethanol precipitation showed that hot- and cold-saline solutions remove more amino acids than do hot and cold water.

With colloidal-iron precipitation the extraction is only effective in the presence of saline. The chromatograms show that hot saline is more effective in extracting amino acids than is cold saline.

The chromatograms obtained using the dialysed extracts show that, although there is no difference between the extractions with hot saline and hot water, they both give better extractions than do cold solutions.

Table 1. *Comparison of the different media used for extraction of non-protein nitrogen (NPN) from pea seeds*

Total N/g. of seeds was 11·7 mg. Extractions are expressed as mg./g. green seeds (from triplicate estimations).

		NPN in extracts after				
Extracting medium	Total extractable N	Trichloro-acetic acid	Ethanol	Tungstic acid	Colloidal iron	Dialysis
Cold saline buffer	9·0	4·5	4·2	3·6	4·4	4·0
Cold water	9·0	4·2	3·2	2·8	—	4·1
Hot saline buffer	7·6	5·7	5·5	4·7	5·3	4·8
Hot water	7·2	4·6	3·9	3·0	—	4·7

Table 2. *Free amino acids after action of extracting agents and protein precipitants*

	mg. carboxyl N/g. green seeds (from triplicate estimations)				
Extracting medium	Trichloro-acetic acid	Ethanol	Tungstic acid	Colloidal iron	Dialysis
Cold saline buffer	3·1	2·0	2·8	2·1	2·1
Cold water	2·6	1·6	2·3	—	2·3
Hot saline buffer	3·7	3·0	3·9	2·9	3·0
Hot water	3·1	2·1	2·3	—	3·2

DISCUSSION AND SUMMARY

Green seed peas were extracted with hot and cold saline buffer and with hot and cold water. The protein was removed from these extracts by trichloroacetic acid, ethanol, tungstic acid and colloidal iron; diffusates were also prepared by dialysis of the extracts. Cold water and saline extracted more nitrogen-containing compounds, but the best yields of non-protein nitrogen were obtained by hot-saline extraction followed by trichloroacetic acid precipitation. Ethanol and colloidal iron gave good recoveries after a similar extraction, but the amounts yielded by tungstic acid precipitation and by dialysis were less. This action of precipitants is similar to that observed by Steward & Street (1946) in their survey of the soluble-nitrogen fractions of the potato tuber.

The amount of free amino acid was high in the hot-saline extract after trichloroacetic acid precipitation but the highest values were obtained after tungstic acid precipitation.

Paper chromatography of the extracts showed the presence of numerous amino acids. Tungstic acid filtrates are unsuitable for chromatography.

It is suggested that, for a complete extraction of non-protein nitrogen from peas, hot saline-buffer extraction followed by trichloroacetic acid precipitation is the best procedure. Excess trichloroacetic acid may be easily removed by extraction with ether. The procedure of next choice would appear to be the use of ethanol.

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The Liberation of Aspartic Acid during the Acid Hydrolysis of Proteins

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Comparatively little is known about the relative rates of hydrolysis of the different peptide bonds in proteins, or about the effect on these rates of changes in hydrolytic conditions (cf. Acher, Jutisz & Fromageot, 1950; Desnuelle & Casal, 1948). Partridge & Davis (1950) found that when a number of proteins were hydrolysed with boiling weak acids, aspartic acid was liberated in the free state much sooner than other amino acids, and later Blackburn (1950*a*) showed that aspartic acid was also preferentially liberated during hydrolysis of proteins with dilute solutions of strong acids at the boil.

The present paper is concerned with the liberation of aspartic acid under various conditions of acid hydrolysis, and the extent to which this liberation occurs independently of the splitting of other linkages. A mechanism for the liberation is suggested.

MATERIALS AND METHODS

A '56s' quality virgin wool (Blackburn, Consden & Phillips, 1944) was used in the first experiments. As the supply of this wool became exhausted, a '64s A' quality Australian wool was cleaned in a similar manner and used in the later experiments. Crystallized bovine plasma albumin was obtained from Armour Laboratories.

Partial hydrolysis and estimation of free amino acids. The wool or other protein was hydrolysed with HCl (100 ml./g.) under the conditions stated below, any undissolved wool filtered off and excess of acid removed by repeated distillation *in vacuo* below 40°, with successive additions of water. The hydrolysates were examined on phenol–NH_3, collidine, or two-dimensional collidine/phenol–NH_3 chromatograms, the concentration of the free amino acids being estimated by visual observation of the size and intensity of the amino acid spots on the chromatogram. The ×'s of Table 1 have the significance attached to them by Consden, Gordon, Martin & Synge (1947) and Consden, Gordon & Martin (1949), and give a semiquantitative estimate of the amounts of the amino acids. Proline was detected by the isatin reaction (Acher, Fromageot & Jutisz, 1950) and cystine by oxidation to cysteic acid with hydrogen peroxide (Dent, 1948). The concentration of free aspartic acid in some of the hydrolysates was measured by the method of Blackburn & Robson (1953). The peptides present in the partial hydrolysates had very small R_F values and were far removed from the aspartic acid spot, and other free amino acids were only present in traces. It was therefore possible to estimate the aspartic acid on one-dimensional phenol–NH_3 chromato-

grams of the hydrolysates. Determination of the aspartic acid by 'spot dilution' analysis (Polson, Mosley & Wyckoff, 1947) gave values of the same order of magnitude as those obtained by the radiochemical method. The figures in Table 2, however, are regarded as giving the order rather than an exact value for the free aspartic acid in solution.

Deamidation of wool. To remove the amide nitrogen, wool (1 g.) was either treated with nitrous acid in 2 N-HCl for 24 hr. (Plimmer, 1925; Fraenkel-Conrat, Cooper & Olcott, 1945), or treated with 100 ml. of 0·05 M cetylsulphonic acid (prepared according to McBain & Williams, 1933) at 65° for 24 hr. (Steinhardt & Fugitt, 1942).

Isolation of peptides and end-group estimations. Large polypeptides present in partial hydrolysates were isolated as follows (cf. Partridge & Davis, 1950). The solution was neutralized with Li_2CO_3 and 3 vol. of ethanol were added. After standing in the refrigerator for some time the precipitated peptides were removed by centrifuging. Amino-N (Van Slyke) determinations showed that these peptides had long chains. The dinitrophenylpeptides (DNP-peptides) were prepared as described previously (Blackburn, 1950*b*), insoluble peptides being treated with 1-fluoro-2:4-dinitrobenzene (FDNB) for 24 hr. and soluble peptides for 4 hr. To determine the *N*-terminal amino acids in partial hydrolysates, the hydrolysate was made alkaline with $NaHCO_3$, treated with FDNB, diluted and extracted with ether, made 5·7 N in HCl and refluxed for 17 hr. after removing dissolved ether by warming. The DNP-amino acids liberated on acid hydrolysis were separated on buffered silica columns (Blackburn, 1949).

RESULTS

The liberation of free amino acids

Table 1 shows the relative amounts of free amino acids liberated during the hydrolysis of wool under various conditions. During 4 hr. hydrolysis with 0·1 N-HCl at the boil, only aspartic acid is liberated in the free state. This preferential liberation of aspartic acid is not confined to HCl, for 0·1 N-H_2SO_4 at the boil acts similarly. With increasing concentration of acid (HCl up to N), other amino acids are liberated, glycine being one of the first. Serine is liberated readily, valine, leucine and threonine slowly. The order is roughly that of increasing length of side chain of the amino acid; a similar order is observed when wool is hydrolysed with concentrated HCl at 37°. Cystine and proline are not liberated very readily. The cystine content of the undissolved residue obtained when wool is partially hydrolysed with dilute acid shows a marked increase above that of the original wool, e.g. after boiling with 0·5 N-HCl for 8 hr. the residue amounted to 17 % of original weight of the wool and had increased in cystine content by 67 %. Similar increases have been observed when wool or other proteins are digested with enzymes (cf. Blackburn, 1950*b*). This slow liberation of cystine from the protein is probably due to the fact that four peptide linkages must be broken before cystine is set free, as compared with two for other amino acids.

Table 1. *The liberation of free amino acids during the hydrolysis of wool*

For significance of '×' see Consden, Gordon, Martin & Synge (1947).

Conditions of hydrolysis		Time of hydrolysis (hr.)	Aspartic acid	Glutamic acid	Glycine	Alanine	Valine	Leucine	Serine	Threonine	Basic amino acids
Reagent	Temp.										
0·1 N-HCl	Boiling	1	× ×	·	·	·	·	·	·	·	·
0·1 N-HCl	Boiling	3	× × ×	·	·	·	·	·	·	·	·
0·1 N-HCl	Boiling	5	× × ×	·	×	·	·	·	·	·	·
0·25 N-HCl	Boiling	1	× ×	·	×	·	·	·	·	·	·
0·25 N-HCl	Boiling	3	× × ×	·	× ×	×	·	×	×	·	·
0·5 N-HCl	Boiling	1	× ×	×	× ×	×	×	× ×	·	·	·
0·5 N-HCl	37°	144	·	·	?	×	?	×	·	·	·
0·5 N-HCl	50°	72	·	·	× ×	·	·	·	·	·	·
N-HCl	Boiling	2	× × ×	× ×	× ×	×	·	×	× ×	·	·
N-HCl	37°	72	·	·	× ×	·	·	·	?	·	·
N-HCl	37°	144	?	?	× ×	·	?	·	·	·	·
N-HCl	50°	72	× ×	× ×	× × ×	× ×	?	× ×	· ×	?	·
5 N-HCl	37°	24	?	×	× ×	×	?	×	× ×	×	·
Conc. HCl	37°	24	× ×	×	× ×	·	·	·	× ×	·	·
Conc. HCl	37°	48	× ×	×	× ×	×	·	·	× ×	·	·
0·1 N-$Ba(OH)_2$	Boiling	1	×	× ×	× ×	× ×	·	· ×	× ×	·	×
0·25 N-$Ba(OH)_2$	Boiling	1	× ×	×	× ×	×	·	? ×	× ×	·	× ×

Table 2. *The free aspartic acid and N-terminal residues liberated during the hydrolysis of bovine plasma albumin with dilute acid at the boil*

		Time of hydrolysis (hr.)				
		2	4	6	8	24
Free aspartic acid (μmoles/g. protein) after hydrolysis with (*a*) 0·1 N-HCl and (*b*) 0·25 N-HCl	(*a*)	70	181	198	338	.
	(*b*)	163	257	400	666	.
N-terminal residues (μmoles/g. protein) after hydrolysis with 0·1 N-HCl						
Aspartic acid		.	53	.	132	270
Glutamic acid		.	26	.	37	82
Serine		.	9·5	.	16	53
Threonine		.	28	.	42	102
Glycine		.	Present	.	Present	Present
Alanine		.	Present	.	Present	Present
Valine		.	Present	.	Present	Present
Leucine + isoleucine		.	Present	.	Present	Present

Table 3. *The N-terminal residues of long-chain peptides from protein hydrolysates*

N-terminal residues (μmoles/g. DNP-peptide)	Wool	Bovine plasma albumin
Aspartic acid	3·5	3·9
Glutamic acid	6·4	13·6
Serine	19·9	5·9
Threonine	8·5	12·9
Glycine	Present	Present
Alanine	Present	Present
Valine	4·9	Present
Leucine + isoleucine	11·7	Present

As some of the aspartic acid residues in proteins are combined with ammonia as asparagine, it was of interest to determine the effect that removal of the amide groups would have on the liberation of aspartic acid. Wool was therefore deamidated with HNO_2 in HCl, or partially hydrolysed under conditions (cetylsulphonic acid at 65°; concentrated HCl at 37° for 1 or 2 days; or 0·1 N-HCl at the boil 24 hr.) where the amide-N is rapidly removed (cf. Steinhardt & Fugitt, 1942; Gordon, Martin & Synge, 1941). When these wools or partial hydrolysates were boiled with 0·1 N-HCl for 4 hr., chromatographic examination of the resulting hydrolysates showed that aspartic acid was still liberated at a rate exceeding that of other amino acids. Removal of the amide groups thus has no effect on the preferential liberation of aspartic acid.

Table 2 shows that, in addition to the liberation of aspartic acid, a large number of different *N*-terminal residues are formed when bovine plasma albumin is hydrolysed with 0·1 N-HCl for various times. No correction factor has been applied to these figures for the destruction of DNP-amino acids during hydrolysis, since its magnitude is rather uncertain and the relative rather than the absolute values are of importance to the present argument.

Two peptide linkages must be hydrolysed in order to set free an aspartic acid residue in a peptide chain; the first one to be hydrolysed may involve either the α-amino or α-carboxyl group of the residue and the partial hydrolysate should then contain relatively large amounts of either aspartyl or aspartic acid peptides. In an experiment to obtain evidence on this point the long-chain peptides from a partial hydrolysate of Australian '64s A' wool (7 g.) with 0·1 N-HCl for 8 hr. at the boil were precipitated as described above and converted into their DNP derivatives (0·141 g.). In a parallel experiment bovine plasma albumin (250 mg.) boiled for 4 hr. with 0·1 N-HCl gave 157 mg. of DNP-peptide. The free amino groups of both these peptide fractions were then determined (Table 3). The most noteworthy point is that the amount of *N*-terminal aspartic acid present is small compared with those of other *N*-terminal residues. The long-chain peptides represent an intermediate stage in the breakdown of protein to short peptides and at this stage, i.e. after 4 or 8 hr., aspartic acid is still being liberated preferentially compared with the other amino acids. These results may therefore indicate that the peptide linkage involving the α-carboxyl group of aspartic acid is the first to be broken. Desnuelle & Bonjour (1952) suggested that a partial hydrolysate of protein with oxalic acid might contain seryl and threonyl peptides, and that more aspartyl peptides than aspartic acid peptides should be present. In dilute HCl hydrolysates, seryl and threonyl peptides are certainly present but the amount of aspartyl peptides seems to be small. Table 2 and amino-N determinations on the hydrolysates showed that for every aspartic acid residue set free several other peptide linkages involving other amino acids are broken. The liberation of aspartic acid during dilute acid hydrolysis has thus only a limited specificity.

DISCUSSION

There is a marked difference in the rate at which free aspartic acid is liberated during hydrolysis with concentrated hydrochloric acid at 37° and dilute hydrochloric acid at the boil. Partridge & Davis (1950) considered that during the hydrolysis of proteins with weak acids such as oxalic acid, free carboxyl groups were released from asparagine and glutamine side chains at an early stage. These carboxyl groups then acted as proton donors to promote the hydrolysis of peptide linkages involving first the α-carboxyl groups and then the α-amino groups, liberating free aspartic or glutamic acid. The release of aspartic acid at an earlier stage than glutamic acid was attributed to the lower pK_1 and pK_2 values of aspartic acid as compared with glutamic acid. On this mechanism it would be expected that cysteic acid residues in a protein, which differ from aspartic acid residues in having a sulphonic group at the end of the chain instead of a carboxyl group, would be set free earlier than aspartic acid during hydrolysis with dilute acids. In fact, cysteic acid was liberated from wool oxidized with performic acid (Blackburn & Lowther, 1951) at a fairly early stage but at a slower rate than aspartic acid. Sanger & Tuppy (1951) similarly observed that large amounts of free aspartic acid, but only small amounts of free cysteic acid, were present in the digest when oxidized insulin was boiled with 0·1 N hydrochloric acid.

Partridge & Davis further suggested that a similar type of argument should apply to the free carboxyl group at the end of a peptide chain. It is noteworthy, however, that when lysozyme, whose molecule contains a single amino acid chain with a *C*-terminal leucine residue (Thompson, 1952) was boiled with 0·1 N hydrochloric acid, the short-chain amino acids, not leucine, were the first to be liberated after aspartic acid.

Since the liberation of free glutamic acid does not show anything like the variation with changes in hydrolytic conditions shown by aspartic acid, some mechanism must operate which is applicable to aspartic acid and not to glutamic acid. Liberation of glutamic acid with subsequent ring closure to pyrrolidonecarboxylic acid does not seem to occur to a significant extent, since when a partial hydrolysate of wool with 0·1 N hydrochloric acid for 4 hr. was further hydrolysed for 24 hr. with 5 N hydrochloric acid after removal of insoluble protein material and examined chromatographically, glutamic acid was not present in amount noticeably greater than other amino acids. In addition, the preferential liberation of aspartic acid on boiling with dilute hydrochloric acid from wool already partially hydrolysed with concentrated hydrochloric acid at 37° means that the mechanism must apply to relatively short-chain peptides in addition to intact proteins.

One possible mechanism is that ring closure occurs between the β-carboxyl group of aspartic acid and the adjacent carboxyl group of the peptide linkage, fission of the peptide linkage then occurring (Fig. 1*a*). Barker (1953) observed that *N*-acetyl- or *N*-benzoyl-aspartic acid with acetic anhydride formed an internal anhydride rather than an oxazolone. Ring closure could possibly occur also in the other direction (Fig. 1*b*).

Baker & Ollis (1949) found that β-benzamido-*iso*valeric acid underwent ring closure on treatment with acetic anhydride to give 6-oxo-2-phenyl-4:4-dimethyl-5:6-dihydro-1:3-oxazine, and suggested that aspartic acid derivatives might react in a similar manner. The relative rates of the cyclization and hydrolysis reactions may be such that the ring compound never appears in solution (cf. Sanger, 1952). At 37°, acid of low concentration (0·1–0·5 N hydrochloric acid) liberates glycine and serine preferentially, indicating that the principal factor in the formation of free aspartic acid at the boil with dilute acid is the higher temperature. This fact would suggest that a ring closure such as the above is favoured by a rise in temperature. Other instances where aspartic acid or asparagine residues in peptide linkage show exceptional lability (Waelsch, 1952; Weygand & Junk, 1951; Thompson, 1953)

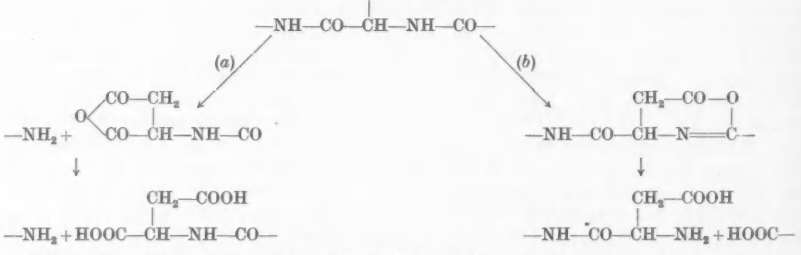

Fig. 1. Suggested mechanisms for preferential release of aspartic acid from proteins.

are probably due to the presence of a reactive residue such as serine adjacent to the aspartic acid or asparagine residue (Thompson, 1953).

SUMMARY

Aspartic acid is preferentially liberated during hydrolysis of proteins with hot dilute acids but not with concentrated HCl at 37°. The specificity of the reaction and its mechanism are discussed.

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Effects of Nicotine on the Pyruvic Oxidase System in Brain

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The pharmacology of nicotine has been extensively studied, but comparatively little is known about its mode of action in the biochemical sense.

Nicotine, in common with other pyridine derivatives (Dickens, 1936*a*), inhibits the Pasteur effect and retards the enzymic degradation of cozymase (Baker, Fazekas & Himwich, 1938; McIlwain, 1950). It may interfere with coupled phosphorylation (Case & McIlwain, 1951) but does not affect the hexokinase reaction (Fahmy & Walsh, 1954). Nicotine is particularly toxic to nerve cells (Langley & Dickinson, 1889, 1890*a*, *b*); the toxicity towards living organisms parallels the degree of development of the nervous system (Greenwood, 1890), and the effects on nerve tissue are analogous to those resulting from oxygen deprivation (McIndoo, 1916; Wilson, 1928).

It is well known that nicotine inhibits cellular respiration and, since nerve is uniquely dependent on carbohydrate (Dickens, 1936*b*) and is unable to metabolize nicotine (Werle & Müller, 1941; Miller & Larson, 1953), it seemed probable to us that the toxicity of nicotine towards nerve cells might be attributable to its ability to block essential steps in the oxidative metabolism of carbohydrate. In the present work we have made an analytical study of the effects of nicotine on the oxidation of pyruvate through the citric acid cycle.

MATERIALS AND METHODS

Substrates. Neutral solutions were prepared freshly as required from pure, crystalline materials. Sodium pyruvate was prepared as described by Price & Levintow (1952).

Homogenates. Glass homogenizers of Potter & Elvehjem's type were used. Tissues (rat-brain cerebral cortex, pigeon cerebral hemispheres or rat-kidney cortex) were homogenized in Krebs's phosphate–saline, with cooling, to give suspensions containing 100 mg. tissue/ml.

Manometric methods. O_2 uptakes were measured in the Warburg apparatus at 37° and pH 7·4. Gas phase, air. Shaking, 100 strokes/min. The centre wells contained 20% (w/v) KOH (0·2 ml.). Substrates were added to the main compartments from the side arms after 30 min. equilibration.

Citrate was determined by the method of Natelson, Pincus & Lugovoy (1948), using a Unicam Quartz Spectrophotometer.

Acetate was determined by the method of Bartley (1953).

EXPERIMENTAL AND RESULTS

Effects of nicotine on respiration of brain tissue with glucose and pyruvate as substrates

Oxygen uptakes by rat-brain homogenate in the presence and absence of nicotine with either glucose (0·02 M) or pyruvate (0·02 M) as substrate are compared in Table 1. The values obtained in different experiments for respiratory inhibition by nicotine are consistent and are constant throughout the experimental period (2 hr.).

The results show that the oxidation of pyruvate is more sensitive towards nicotine than is that of glucose.

Effect of nicotine on the oxidation of succinate in brain

The effect of nicotine on the oxidation of sodium succinate (0·02 M) by rat-brain homogenate was determined in the presence of sodium arsenite (0·0033 M) which, according to Green, Loomis & Auerbach (1948), inhibits oxidation of succinate in the cyclophorase system beyond the fumarate → malate stage, although the actual site of inhibition by arsenite is probably at the later, oxaloacetate or pyruvate stage (Long, 1945). Under our experimental conditions, arsenite (0·0033 M) reduced the oxygen uptake by rat brain with succinate as substrate from 400 to 120 μl./100 mg. tissue/1·5 hr. Nicotine at 0·0075 M inhibited by 5–6% and at 0·03 M by 7–8%.

These results are in close agreement with those obtained (unpublished work) when succinic dehydrogenase activities of muscle preparations were measured by the triphenyltetrazolium bromide method, previously described (Fahmy & Walsh, 1952).

Effect of nicotine on the oxidation of fumarate to oxaloacetate in kidney

In spite of the fact that brain contains fumarase (Breusch, 1938) and malic dehydrogenase (Green, 1936), oxygen uptake by brain homogenate is not increased by addition of fumarate. Kidney tissue was therefore used.

The effect of nicotine on oxygen uptake by rat-kidney homogenate with sodium fumarate (0·02 M) as substrate was measured in the presence of methylene blue and cyanide, which binds oxaloacetate (Green & Williamson, 1937), thereby isolating the step, fumarate → oxaloacetate (Mann & Quastel, 1941).

The results (Table 2) show that nicotine inhibits this system but has less effect than it has on the pyruvic oxidase system as a whole.

Table 1. *Effects of nicotine on glucose and pyruvate oxidation in brain*

Each vessel contained rat-brain homogenate (100 mg. tissue) in Krebs's phosphate–saline, substrate (0·02 M), ±nicotine, total vol. 2·5 ml.; O_2 uptakes measured at 37° and pH 7·4.

Glucose					Sodium pyruvate			
	O_2 (μl.)				O_2 (μl.)			
Expt.		Control	Nicotine (0·0075 M)	Inhibition (%)	Expt.	Control	Nicotine (0·0075 M)	Inhibition (%)
1	1st hr.	109	98	10	6	147	124	16
	2nd hr.	96	86	10		110	90	18
2	1st hr.	118	104	12	7	126	108	14
	2nd hr.	102	91	11		100	86	14
3	1st hr.	126	112	11	8	136	116	15
	2nd hr.	109	98	10		96	82	15
			(0·03 M)				(0·03 M)	
4	1st hr.	120	95	20	9	139	100	28
	2nd hr.	100	77	23		115	79	31
5	1st hr.	110	86	22	10	156	105	33
	2nd hr.	88	70	21		128	84	34
					11	144	99	31
						110	73	34

Table 2. *Effect of nicotine on oxidation of fumarate to oxaloacetate in kidney*

Each vessel contained rat-kidney homogenate (100 mg. tissue) in Krebs's phosphate–saline, sodium fumarate (0·02 M), NaCN (0·08 M), methylene blue (0·016 %), ±nicotine, total vol. 2·7 ml.; O_2 uptakes measured at 37° and pH 7·4.

Expt.	O_2 (μl./100 mg. tissue/1·5 hr.)	Nicotine (0·0075 M)	Inhibition (%)
1	70	70	0
2	76	75	1
3	72	71	1
4	84	84	0
		(0·03 M)	
5	80	70	12
6	89	77	13
7	73	64	12
8	90	80	11

Table 3. *Effect of nicotine on oxidative decarboxylation of α-oxoglutarate in brain*

Each vessel contained rat-brain homogenate (100 mg. tissue) in Krebs's phosphate–saline, sodium α-oxoglutarate (0·02 M), sodium malonate (0·033 M), ±nicotine; total vol. 2·6 ml.; O_2 uptakes measured at 37° and pH 7·4.

Expt.	O_2 (μl./100 mg. tissue/1·5 hr.)	Nicotine (0·0075 M)	Inhibition (%)
1	90	86	5
2	96	90	6
3	92	87	5
		(0·015 M)	
4	86	77	10
5	103	92	11
6	110	97	12
		(0·03 M)	
7	100	85	15
8	98	82	16
9	104	86	17
10	106	90	15

Effect of nicotine on the oxidation of citrate to α-oxoglutarate in liver

Nicotine (0·03 M) had no effect on oxygen uptake at 37° and pH 7·4 by pigeon-liver homogenate (brain tissue being unsuitable) with sodium citrate (0·02 M) as substrate in the presence of sodium arsenite (0·0033 M) which serves to isolate the step, citrate → α-oxoglutarate (Krebs & Johnson, 1937). Oxygen uptakes of 150–200 μl./100 mg. tissue/1·5 hr. were obtained.

Effect of nicotine on oxidative decarboxylation of α-oxoglutarate in brain

The effect of nicotine on the step, α-oxoglutarate → succinate was determined by measuring oxygen uptake by rat-brain homogenate with sodium α-oxoglutarate (0·02 M) as substrate in the presence of sodium malonate (0·033 M).

The results (Table 3) show that the inhibiting effect of nicotine on this system is of the same order as that on the oxidation of fumarate to oxaloacetate.

Effect of nicotine on citrate synthesis in brain and kidney

The effect of nicotine on the synthesis of citrate from pyruvate and oxaloacetate by rat brain and kidney have been determined in the presence of inhibitors which prevent the metabolism of citrate. Fluoroacetate was used in experiments with kidney. Since brain tissue, unlike that of kidney, does not form the inhibitor from fluoroacetate (Peters, 1953), $BaCl_2$ was used in experiments with brain (Wieland & Rosenthal, 1943).

Mixtures (3·0 ml.), with and without nicotine, containing tissue homogenate (≡300 mg. tissue), pyruvate (0·02 M), oxaloacetate (0·02 M) and either $BaCl_2$ (0·033 M) or fluoroacetate (0·01 M) were shaken in the Warburg apparatus at 37° and pH 7·4. Samples (1·0 ml.) were withdrawn at zero time and after 1 hr. for determination of citrate, having heated to destroy citrate-simulating substances as recommended by Krebs & Eggleston (1948). In the experiments with kidney, an initial incubation period (15 min.) was allowed to permit fluorocitrate to be formed before adding the substrates.

The results (Table 4) show that the inhibition (40 %) of citrate formation by nicotine (0·03 M) is greater than that of any of the other steps in the citric acid cycle, and that brain and kidney tissue are, in this respect, equally affected.

In experiments carried out on the synthesis of citrate from acetate by baker's yeast, following the procedure described by Ryman & Walsh (1954), nicotine had no appreciable effect, thereby suggesting that nicotine, unlike cocaine, does not inhibit the condensing enzyme.

Effect of nicotine on oxidative decarboxylation of pyruvate

The effects of nicotine on the amount of acetate formed from pyruvate in dialysed pigeon-brain homogenates (Banga, Ochoa & Peters, 1939) have been determined in the system as used by Coxon, Liébecq & Peters (1949). The results (Table 5) show that nicotine inhibits the pyruvic dehydrogenase system to the same extent as it inhibits the synthesis of citrate from pyruvate. It would appear, therefore, that the inhibitory action of nicotine on the

Table 4. *Effects of nicotine on citrate synthesis in brain and kidney homogenates*

Mixtures (3·0 ml.), containing in Krebs's phosphate–saline, tissue (300 mg.), sodium pyruvate (0·02 M), sodium oxaloacetate (0·02 M), either $BaCl_2$ (0·033 M; brain) or sodium fluoroacetate (0·01 M; kidney), ±nicotine, were shaken in air at 37° and pH 7·4. Citrate synthesized = 1 hr. value − 0 hr. value.

	Brain			Kidney		
	Citrate (μg./300 mg. tissue/hr.)			Citrate (μg./300 mg. tissue/hr.)		
Expt.	Control	Nicotine (0·0075 M)	Inhibition (%)	Control	Nicotine (0·0075 M)	Inhibition (%)
1	189	151	20	870	736	15
2	199	161	19	825	680	18
3	150	122	19	850	697	18
		(0·015 M)			(0·015 M)	
4	149	99	34	900	612	32
5	201	140	30	814	570	30
6	170	119	30	730	508	30
		(0·03 M)			(0·03 M)	
7	179	100	44	680	415	39
8	220	132	40	780	468	40
9	180	108	40	912	538	41

Table 5. *Effects of nicotine and vitamin B_1 on pyruvic dehydrogenase in dialysed pigeon-brain homogenate*

Each flask contained in 0·08 M potassium phosphate buffer (2·5 ml.), tissue (200 mg.), ATP (1 μmole), $MgCl_2$ (4 μmoles), sodium pyruvate (30 μmoles), ±nicotine, ±vitamin B_1. Acetate formed at 37° and pH 7·4 = 0·5 hr. value − 0 hr. value.

		Acetate (μg./200 mg. tissue/0·5 hr.)				Activation (%)	Inhibition (%)	
Expt.	Molar concentration of nicotine and/or vitamin B_1	Control	Nicotine	Vitamin B_1	Nicotine + vitamin B_1	Vitamin B_1	Nicotine	Nicotine + vitamin B_1
1	0·0075	560	440	590	460	5	21	22
2		480	400	520	432	8	17	17
3		510	420	525	430	3	18	18
4	0·015	600	420	615	425	3	30	31
5		560	405	565	396	1	29	30
6		620	415	630	435	2	33	31
7		510	376	500	355	−2	28	29
8	0·03	547	322	580	360	6	41	38
9		501	300	520	302	4	40	42
10		602	360	600	365	0	40	39
11		520	300	516	300	0	42	42

pyruvic oxidase system is the result of a specific inhibition of pyruvic dehydrogenase.

We have included in our experiments a study of the effect of nicotine on this system in the presence of added vitamin B_1. Vitamin B_1 was added to give a final concentration equal to that of the nicotine. The results show that addition of vitamin B_1 does not protect the enzyme from the inhibitory action of nicotine.

It is of interest to report here that addition of vitamin B_1 to homogenates, prepared from the brains of pigeons which were used immediately after purchase, increased acetate formation by as much as 20 %. On the other hand, vitamin B_1 had little or no effect when added to homogenates prepared from pigeons which had been kept in our own animal house for several days.

DISCUSSION

Preliminary experiments with yeasts (unpublished data), analogous to those employed in the study of the action of cocaine on cellular metabolism (Ryman & Walsh, 1951, 1952), have confirmed the reports of earlier workers (reviewed by Enders & Windisch, 1947) that nicotine, while inhibiting respiration, activates fermentation and may even accelerate anaerobic growth.

Himwich & Fazekas (1935, 1936) reported that nicotine inhibited respiration of minced brain with lactate or succinate as substrates but not with either glucose or pyruvate. They concluded that lactic dehydrogenase was probably inhibited. Later, Baker *et al.* (1938), using brain slices, found glucose and pyruvate oxidation to be inhibited

while succinate oxidation was little affected. They reported inhibitions of 49 % with glucose, 76 % with pyruvate and 91 % with lactate by nicotine (0·3 M). In our experiments with homogenates of rat-brain cerebral cortex we have not obtained inhibitions as high as those reported by Baker *et al.*, but we have obtained definite and consistent inhibitions of respiration with glucose as substrate and consistently higher inhibitions with pyruvate. These results emphasize that the site of action of nicotine in inhibiting carbohydrate oxidation lies in the metabolic path following the pyruvate stage.

Since inhibition of succinate oxidation had previously been reported, and in view of the possibility that nicotine might depress oxidation, not by inhibiting any of the enzymes specific to the substrates of the citric acid cycle but by interfering with the electron-transferring mechanism of respiration, we investigated the effect of nicotine on the aerobic oxidation of succinate in the presence of arsenite. Nicotine had but little effect on this system, thus indicating that succinic dehydrogenase and its associated cytochrome system are not appreciably affected. This information completed the evidence that nicotine does not inhibit respiration by interference with the general mechanism of hydrogen and electron transfer. Experiments with other dehydrogenase systems (unpublished work) have shown that nicotine is not a general inhibitor of systems involving either cozymase or the flavoproteins.

The experiments with other isolated steps of the citric acid cycle show that nicotine (0·03 M) has no appreciable effect on the oxidation of citrate to α-oxoglutarate, and that the inhibitions of the enzymes involved in the oxidations of fumarate to oxaloacetate and of α-oxoglutarate to succinate are insufficiently great to account for the effect of nicotine on pyruvate oxidation.

While the synthesis of citrate from acetate by baker's yeast is not affected by nicotine, citrate synthesis in brain and kidney homogenates, with pyruvate as the source of active acetate, is inhibited, nicotine (0·03 M) inhibiting by 40 %. It is of interest to note that there is no difference between brain and kidney tissue in the effects of nicotine on citrate synthesis.

The results of investigations into the effect of nicotine on the oxidative decarboxylation of pyruvate, using dialysed brain homogenate to isolate the step, pyruvate → acetate, confirm that the inhibitory action of nicotine on pyruvate oxidation may be attributed primarily to a specific inhibition of pyruvic dehydrogenase.

Although nicotine inhibits choline acetylase (Fahmy, Ryman & Walsh, 1954), it has no appreciable effect either on sulphanilamide acetylation in liver or on citrate synthesis from acetate in yeast. It would appear, therefore, that nicotine is not a general inhibitor of all reactions in which active acetate is involved.

With regard to any discussion concerning the mechanism for the inhibitory action of nicotine on the oxidative decarboxylation of pyruvate, it is relevant to compare the behaviour of this system with that concerned in the analogous oxidative decarboxylation of α-oxoglutarate. As pointed out by Green (1952), both systems require the same cofactors but, as our experiments show, the α-oxoglutarate system is much less sensitive towards nicotine. It is also much less sensitive towards deficiency of vitamin B_1 (Peters, 1953).

Since some of the pharmacological effects of nicotine are antagonized by vitamin B_1 (Unna & Pick, 1944; Jalón & Bayo, 1946; Haimovici & Pick, 1946; Pick & Unna, 1946), and there is some structural resemblance between the two compounds, one might have expected to find a biochemical antagonism between them. The results obtained in the present work, however, show that addition of vitamin B_1 to the pyruvic dehydrogenase system has no influence on the inhibitory action of nicotine when present in equal concentration. We have also observed, in our preliminary experiments with yeasts, that the stimulating effects of nicotine and of vitamin B_1 on fermentation are additive and apparently independent of one another.

It is of interest to note that barbiturates also block aerobic pyruvate metabolism by inhibiting pyruvic dehydrogenase and to a lesser extent the oxidative decarboxylation of α-oxoglutarate (Persky, Goldstein & Levine, 1950).

SUMMARY

1. Nicotine inhibits the oxidation of pyruvate by brain tissue more strongly than that of glucose.

2. Nicotine inhibits pyruvate oxidation in brain by specifically inhibiting pyruvic dehydrogenase, other oxidative steps being less sensitive.

3. Addition of vitamin B_1 does not modify the effect of nicotine on the pyruvic dehydrogenase system in dialysed pigeon-brain homogenate.

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Incorporation of Radioactive Phosphate into Lipids and Pentosenucleic Acid of Cat Brain Slices. The Effect of Inorganic Ions

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It is well known that an increase in the concentration of potassium ions, or a decrease in the concentration of calcium ions, causes an increase in the oxygen consumption and aerobic glycolysis of brain slices. There is also a decrease in the anaerobic glycolysis. More recently, McIlwain (1952*a*) and Gore & McIlwain (1952) have shown that if the concentration of K^+ in the medium is increased or the concentration of Ca^{2+} is decreased, respiring slices of guinea pig brain are unable to maintain their concentration of phosphocreatine and other labile phosphorus compounds.

Since it has been shown previously that the incorporation of inorganic phosphorus labelled with ^{32}P into the pentosenucleic acid (DeLuca, Rossiter & Strickland, 1953), phospholipid (Strickland, 1954), and other protein-bound phosphorus compounds (Findlay, Strickland & Rossiter, 1954) of respiring brain slices is dependent upon oxidative phosphorylation mechanisms within the slice, it seemed of interest to determine the effect of increasing the concentration of K^+ or decreasing the concentration of Ca^{2+} on the incorporation of inorganic ^{32}P into these phosphorus-containing compounds. Since the addition of NH^{4+}, L-glutamate, or L-glutamine, also causes a decrease in the concentration of phosphocreatine in respiring brain slices (McIlwain, 1951, 1952*a*; Gore & McIlwain, 1952), the effect of these substances was also investigated.

A preliminary account of some of these experiments has appeared (Rossiter & Findlay, 1954).

METHODS

Slices of cat brain were incubated in Warburg flasks containing Krebs–Ringer bicarbonate, glucose and [^{32}P]-phosphate. The vessels were filled with a mixture of 95% O_2 and 5% CO_2 and incubated in a bath at 37·2° for 4 hr., with shaking to ensure proper oxygenation. When inorganic ions were added or omitted, the medium was kept at the same tonicity by adding or omitting the requisite quantity of NaCl. Inorganic constituents were added as the sodium salt. Details of the preparation of tissue and incubation have been described by DeLuca *et al.* (1953).

After the period of incubation, the tissue was homogenized and the acid-soluble P, containing the greater part of the radioactivity, was removed as described by DeLuca *et al.* (1953). The lipid P was extracted and purified as described by Strickland (1952, 1954). The nucleic acids were extracted from the protein residue by the method of Hammarsten (1947) and the PNA nucleotides were further purified by two magnesia treatments. For brain slices the mixed nucleotides so obtained have a specific activity within the range given by the individual nucleotides separated chromatographically (DeLuca *et al.* 1953). The protein residue contains the residue organic P (ROP) and the 'phosphoprotein' (PP). These fractions were separated as described by Findlay *et al.* (1954). The ROP represents the fraction referred to as 'inositide P' by Logan, Mannell & Rossiter (1952). The fraction is analogous to the 'concomitant P' described by Davidson & Smellie (1952) for liver tissue. Middleton (to be published) has shown that this fraction, like the concomitant P of liver tissue, is comprised of a number of metabolically active P-containing compounds. The incorporation of ^{32}P into the PP fraction of brain was studied *in vivo* by Strickland (1952). More recently, experiments *in vitro* have been reported by Engelhardt & Lissovskaia (1953).

The specific activity of the PP was determined by the method of Ernster, Zetterström & Lindberg (1950), as were the specific activities of the purified lipid P, PNA nucleotides and ROP, after ashing with perchloric acid. In this method both the concentration and the radioactivity of the inorganic P are determined on the same sample. The optical density of the blue colour complex was read with a Beckman Model B Spectrophotometer at 730 mμ. and the radioactivity due to the ^{32}P in the sample was determined on the same coloured solution using an M-6 liquid counter (20th Century Electronics, London, S.E. 27). The counting rate was corrected for background and decay and the specific activities were calculated on the basis of standard count of $8{\cdot}1 \times 10^5$ impulses/min./ml. incubating medium.

RESULTS

Potassium, calcium and ammonium. Table 1 shows that an increase in the concentration of K^+ from the usual 5·9 mM to 124 mM greatly depressed the incorporation of ^{32}P into the lipid P. In other experiments this effect was shown with concentrations of K^+ as low as 29 mM (see Table 9). The omission of Ca^{2+} from the medium caused a smaller reduction in the incorporation. The addition of NH_4^+ to give a final concentration of 29 mM caused a decrease of the same order as 124 mM K^+. A further increase in the concentration of NH_4^+ to 118 mM caused a further decrease in the incorporation.

The effect of changing the concentrations of K^+, Ca^{2+} or NH_4^+ on the incorporation of ^{32}P into the PNA was similar to that observed for the lipid P (Table 2). An increase in the concentration of K^+, the addition of NH_4^+, or the omission of Ca^{2+}, caused a decrease in the incorporation.

Findlay *et al.* (1954) showed that for brain slices respiring in a medium containing [^{32}P]phosphate and glucose, considerable quantities of the ^{32}P were incorporated into the ROP and PP fractions. Table 3 shows the result of an experiment in which the specific activities of these fractions were determined. It can be seen that the specific activities of both the ROP and the PP were considerably greater than the values reported in Tables 1 and 2 for the lipid P and PNA. An increase in the concentration of K^+, or the addition of NH_4^+ to the medium, had a similar inhibitory effect on the incorporation of ^{32}P into the ROP and the PP fractions.

Table 1. *Effect of changing the concentration of* K^+, Ca^{2+} *or* NH_4^+ *on the specific activity of the lipid* P *of slices of cat brain*

Slices incubated for 4 hr. at 37·2° in medium containing glucose (0·011 M) and [^{32}P]phosphate. Counts/min./μg. P.

K^+ (mM)	Ca^{2+} (mM)	NH_4^+ (mM)	Expt. 1	Expt. 2	Expt. 3	Expt. 4
5·9	3·6	0	15·9	20·4	13·4	12·7
124	3·6	0	7·1	5·5	7·2	7·7
5·9	0	0	11·5	15·2	12·4	12·5
5·9	3·6	29	5·2	4·9	5·1	5·8
5·9	3·6	118	2·6	2·0	4·5	5·7

Table 2. *Effect of changing the concentration of* K^+, Ca^{2+} *or* NH_4^+ *on the specific activity of the PNA of slices of cat brain*

Slices incubated for 4 hr. at 37·2° in medium containing glucose (0·011 M) and [^{32}P]phosphate. Counts/min./μg. P.

K^+ (mM)	Ca^{2+} (mM)	NH_4^+ (mM)	Expt. 1	Expt. 2	Expt. 3	Expt. 4
5·9	3·6	0	27·2	26·6	21·7	25·3
124	3·6	0	7·8	10·3	16·6	—
5·9	0	0	19·4	—	15·3	21·6
5·9	3·6	29	8·8	11·8	15·1	14·4
5·9	3·6	118	1·9	1·9	—	4·0

Table 3. *Effect of changing the concentration of K^+ or NH_4^+ on the specific activity of the ROP and PP fractions of slices of cat brain*

Slices incubated for 4 hr. at 37·2° in medium containing glucose (0·011 M) and [^{32}P]phosphate. Counts/min./μg. P.

K^+ (mM)	Ca^{2+} (mM)	NH_4^+ (mM)	Residue organic P (ROP)	'Phosphoprotein' (PP)
5·9	3·6	0	432	1390
124	3·6	0	95	680
5·9	3·6	29	107	570
5·9	3·6	118	25	210

Table 4. *Effect of the addition of* L-*glutamate on the specific activity of the lipid* P, *PNA, ROP and PP of slices of cat brain*

Slices incubated for 4 hr. at 37·2° in Krebs–Ringer bicarbonate containing [^{32}P]phosphate. Counts/min./μg. P. The solution of glutamate was prepared by titration of L-glutamic acid with NaOH to pH 7·4.

	Expt. 1		Expt. 2		Expt. 3			
	Lipid P	ROP	Lipid P	ROP	Lipid P	PNA	ROP	PP
Glucose (0·011 M)	6·4	66	11·8	150	22·7	26·0	305	1360
Glutamate (0·01 M)	3·0	37	6·6	59	9·4	—	167	490
Glucose (0·011 M) and glutamate (0·01 M)	3·9	33	6·8	72	14·4	20·1	156	730
No substrate	—	—	—	—	7·7	—	103	670

Table 5. *Effect of the addition of* L-*glutamine on the specific activity of the lipid* P, *PNA, ROP and PP of slices of cat brain*

Slices incubated for 4 hr. at 37·2° in Krebs–Ringer bicarbonate containing [^{32}P]phosphate. Counts/min./μg. P.

	Expt. 1				Expt. 2			
	Lipid P	PNA	ROP	PP	Lipid P	PNA	ROP	PP
Glucose (0·011 M)	29·5	42·4	440	1260	12·1	26·1	162	540
Glucose (0·011 M) and glutamine (0·01 M)	17·9	30·6	366	1040	8·7	16·3	93	400

Table 6. *Effect of adding glucose either before or after the slices on the specific activity of the lipid* P *and PNA of slices of cat brain*

Slices incubated for 4 hr. at 37·2° in Krebs–Ringer bicarbonate containing [^{32}P]phosphate. Counts/min./μg. P.

	Expt. 1		Expt. 2	
	Lipid P	PNA	Lipid P	PNA
No substrate	15·0	23·5	3·3	16·5
Glucose (0·011 M) added before slices	21·3	31·4	11·3	27·5
Slices added before glucose (0·011 M)	16·9	27·0	6·7	20·8

Glutamate and glutamine. Findlay, Rossiter & Strickland (1953) showed that L-glutamate failed to support the incorporation of ^{32}P into the PNA of cat brain slices, and Strickland (1954) reported a similar finding for lipid P. Since McIlwain (1951, 1952*a*) showed that in the presence of L-glutamate or L-glutamine, respiring slices of guinea pig brain failed to maintain their concentration of phosphocreatine, even in the presence of glucose, the effect of glutamate and glutamine on the ^{32}P incorporations was studied further.

Table 4 shows that if the glucose of the incubating medium was replaced by L-glutamate, the incorporation of ^{32}P into the lipid P, PNA, ROP and PP was greatly decreased. Expt. 3 shows that in the presence of glutamate, the incorporation did not differ greatly from that found in the absence of added substrate. The table also shows that the incorporation was very little greater if glucose was added together with the glutamate. The presence of the glutamate inhibited the extra incorporation usually brought about by the addition of glucose.

From Table 5 it can be seen that the addition of L-glutamine had an inhibitory effect on each fraction similar to that produced by L-glutamic acid.

During the experiments with glutamate it was found that, if the slices of brain tissue were allowed to remain at room temperature for a short period in the absence of glucose, the incorporation of ^{32}P into both the lipid P and the PNA was considerably

reduced. Table 6 shows the results of two experiments in which the glucose was added to the medium either before the slices, as was customary, or after the addition of the slices, but before the vessels were filled with O_2–CO_2 gas mixture. It can be seen that for both lipid P and PNA the slices that were exposed to the glucose-free medium for some minutes did not take up the ^{32}P as well as slices not so exposed.

These results recall the earlier report of Dickens & Greville (1933) that a short period of anaerobiosis in the absence of glucose caused a great reduction in the anaerobic glycolysis of brain slices. Subsequently, Macfarlane & Weil-Malherbe (1941) reported that a similar period of anaerobiosis without added substrate caused a reduction in the concentration of easily hydrolysable P, probably mostly adenosine triphosphate (ATP).

DISCUSSION

Potassium. Ashford & Dixon (1935) and Dickens & Greville (1935) found that an increase in the concentration of K^+ in the medium caused an increase in both respiration and aerobic glycolysis, and a decrease in the anaerobic glycolysis, of brain slices from the rabbit and rat, respectively. These findings were confirmed by a number of workers (Canzanelli, Rogers & Rapport, 1942; Lipsett & Crescitelli, 1950). McIlwain (1952*a*) and Gore & McIlwain (1952), in addition to confirming the increase in respiration and aerobic glycolysis, showed that in the presence of excess K^+, brain slices were unable to maintain their concentration of phosphocreatine.

The lower concentration of labile P in slices respiring in a medium containing excess K^+ raises the obvious question of whether the K^+ might stimulate phosphatases responsible for the hydrolysis of labile phosphorus compounds. Gore (1951) found that the adenosinetriphosphatase of brain homogenates was stimulated by an increase in the concentration of K^+. However, results obtained with homogenates of brain tissue are not directly comparable with those obtained with slices, for in both homogenates and extracts an increase in the concentration of K^+ or a decrease in the concentration of Na^+ increases, rather than decreases, anaerobic glycolysis (Racker & Krimsky, 1945; Utter, 1950; Muntz & Hurwitz, 1951*a*). Muntz & Hurwitz (1951*a*) and McIlwain (1952*a*) have summarized the evidence for the view that the effect of increasing the concentration of K^+ is not directly on the phosphatases that break down labile phosphorus compounds.

Lipsett & Crescitelli (1950) suggested that K^+ might increase the oxygen consumption of brain slices by stimulating the K^+-sensitive ATP-phosphopyruvate transphosphorylase reaction. If this were so, the addition of K^+ should increase, rather than decrease, the concentration of labile P and the incorporation of ^{32}P into lipid P and PNA. It is not immediately apparent why an increase in the concentration of K^+ in the medium should cause a depletion of labile P. McIlwain (1952*a*) suggested that in brain slices much of the metabolism is directed towards the maintenance of a high gradient of K^+ between the inside and the outside of the nerve cells. To maintain the same gradient in the presence of a high concentration of K^+ in the medium, a greatly increased expenditure of energy would be necessary. The respiring brain slice can concentrate K^+, presumably by processes similar to those described by Terner, Eggleston & Krebs (1950). This reaction is energy-consuming and could conceivably decrease the quantity of labile P available for other reactions.

Calcium. Dickens & Greville (1935), Canzanelli *et al.* (1942) and Krebs (1950) showed that the oxygen consumption of brain slices was increased when Ca^{2+} was omitted from the medium. McIlwain (1952*a*) and Gore & McIlwain (1952) showed that the omission of Ca^{2+}, like an increase in the concentration of K^+, caused an increase in both oxygen consumption and aerobic glycolysis and a decrease in the ability of the slices to maintain their level of phosphocreatine.

That these antagonistic effects of K^+ and Ca^{2+} on brain slices may have a counterpart *in vivo* is suggested by the observation of Dubner & Gerard (1939) that K^+ enhanced and Ca^{2+} diminished the spontaneous rhythm of the geniculate body of the cat, and by the finding of Libet & Gerard (1939) that K^+ and Ca^{2+} had opposite effects on the olfactory bulb of the isolated frog brain. Another link between the effect of inorganic ions on the metabolism of brain slices and physiological function is provided by the interesting report of Gore & McIlwain (1952) on the response of respiring brain slices to electric stimuli applied *in vitro*. These workers found that the addition of NH_4^+, the omission of Ca^{2+}, or an increase in the concentration of K^+, abolished the metabolic response to stimulation *in vitro*.

Ammonium. Weil-Malherbe (1938) reported that the addition of NH_4^+ to the medium containing brain slices, like an increase in the concentration of K^+, caused an increase in the oxygen consumption and aerobic glycolysis, and a decrease in the anaerobic glycolysis. Gore & McIlwain (1952) confirmed these findings and showed that the addition of NH_4^+, again like an increase in the concentration of K^+, impaired the ability of respiring slices to maintain their level of phosphocreatine. Muntz & Hurwitz (1951*a*) showed that in brain extracts, as opposed to brain slices, the addition of NH_4^+ increased aerobic glycolysis, but had little effect on the breakdown of ATP. A number of

Table 7. *Effect of the addition of* L-*glutamine on the specific activity of the lipid* P, *PNA, ROP and PP of slices of cat brain incubated under different conditions*

Slices incubated for 4 hr. at 37·2° in medium containing glucose (0·011 M) and [^{32}P]phosphate. Counts/min./μg. P.

	Lipid P	PNA	ROP	PP
Expt. 1				
No glutamine, no NH_4^+	29·5	42·4	440	1260
No glutamine, NH_4^+ (29 mM)	8·6	15·3	134	580
Glutamine (0·01 M), NH_4^+ (29 mM)	5·9	11·0	85	600
Expt. 2				
No glutamine, normal K^+	12·1	26·1	162	540
No glutamine, K^+ (29 mM)	8·2	15·3	109	440
Glutamine (0·01 M), K^+ (29 mM)	7·1	17·4	87	390

glycolytic enzymes that are known to be K^+-sensitive, viz. hexokinase, phosphohexokinase and ATP-phosphopyruvate transphosphorylase, are sensitive to NH_4^+ in the absence of K^+ (Muntz & Hurwitz, 1951*b*).

Braganca, Faulkner & Quastel (1953), in their attempt to explain the inhibitory effect of NH_4^+ on the aerobic formation of acetylcholine by brain slices, suggested that the high concentration of NH_4^+ might increase the formation of glutamine from glutamic acid, a reaction demonstrated in brain slices by Krebs (1935*b*) and in cell-free systems by Elliott (1951). The reaction is energy-consuming and in cell-free systems it has been shown to require an adequate supply of ATP. Such a reaction could conceivably decrease the quantity of ATP available for synthetic reactions, such as the formation of acetylcholine and the incorporation of ^{32}P into lipid P, PNA and other P-containing fractions. However, in our experiments neither ATP nor excess glutamine was found to reverse the inhibitory effect of NH_4^+. Table 7 shows the results of two experiments in which glutamine was added. In most instances the low specific activity brought about by the addition of NH_4^+, or the increase in the concentration of K^+, was decreased still further by the addition of glutamine, a result that is not surprising when the inhibitory effect of glutamine, demonstrated in Table 6, is taken into consideration.

The inhibitory effect of NH_4^+ on ^{32}P incorporation is of some interest in relation to the finding that the injection of NH_4^+ can produce convulsions in experimental animals (Sapirstein, 1943; Torda, 1953). It is also known that there is an increase in the concentration of NH_4^+ in the brain after convulsions produced by the administration of convulsant drugs (Richter & Dawson, 1948; Torda, 1953), fluoroacetate (Benitez, Pscheidt & Stone, 1954), or after electrically induced convulsions (Richter & Dawson, 1948).

Glutamic acid and glutamine. Like the addition of NH_4^+ or excess K^+ to the medium, the addition of L-glutamate or L-glutamine causes an increase in aerobic glycolysis and a decrease in the anaerobic glycolysis of brain slices (Weil-Malherbe, 1938). Glutamate and glutamine decreased the ability of guinea pig brain slices to maintain their level of phosphocreatine (McIlwain, 1951, 1952*a*). It is not surprising, therefore, that glutamate was found to inhibit the incorporation of ^{32}P into the P-containing compounds. The preliminary report of Engelhardt & Lissovskaia (1953) also demonstrated that glutamate inhibits the incorporation of ^{32}P into the PP of brain slices.

There is some evidence that L-glutamate is not an energy-yielding substrate for brain tissue (see Weil-Malherbe, 1950, 1952). This would explain the failure of glutamate to support ^{32}P incorporation, but it does not explain the inhibitory effect of glutamate when it is added in the presence of an adequate concentration of glucose. Possible explanations are:

(i) It is known that L-glutamate undergoes oxidative deamination in brain slices to form α-oxoglutarate and ammonia (Krebs, 1935*a*; Weil-Malherbe, 1936). The presence of NH_4^+ has already been shown to inhibit ^{32}P incorporation. However, Krebs (1935*b*) and Weil-Malherbe (1936) have shown that, under the conditions attaining in brain slices, no ammonia accumulates because it reacts with more glutamate to form glutamine. As already seen, this is an energy-requiring reaction that could deplete the slices of their labile P.

(ii) Terner *et al.* (1950) have shown that, in the presence of oxidizable substrate and L-glutamate, the outward diffusion of K^+ from brain slices is reversed. Stern, Eggleston, Hems & Krebs (1949) showed that under these conditions glutamate is taken up by the slice. Both the K^+ and the glutamate enter the brain cells against a concentration gradient. Such an energy-requiring process would be expected to deplete the tissue of labile P. If this hypothesis were true, the addition of glutamate should have an effect on the energy requirements of the slice similar to that produced by an increase in the concentration of K^+. Evidence for this is the finding of Lipsett & Crescitelli (1950) that the stimulatory effect of K^+ on the oxygen consumption

Table 8. *Concentration and specific activity of inorganic* P *of slices and medium before and after incubation under different conditions*

Cat brain slices incubated for 4 hr. at 37·2° in medium containing glucose (0·011 M) and [^{32}P]phosphate.

	Medium		Slice	
	Concn. (μg. P/ml.)	Specific activity (counts/min./μg. P)	Concn. (μg. P/g.)	Specific activity (counts/min./μg. P)
Expt. 1				
Before incubation	104	6 620	241	466
After incubation				
No glutamine, normal K^+	113	4 960	336	3 040
Glutamine (0·01 M), normal K^+	116	4 880	301	2 700
No glutamine, K^+ (29 mM)	114	4 660	285	3 180
Expt. 2				
Before incubation	61	12 100	199	749
After incubation				
No NH_4^+	64	8 000	201	5 260
NH_4^+ (29 mM)	81	7 350	160	4 950

of brain slices was not observed when the medium contained both glucose and glutamate.

The ineffectiveness of glutamic acid as an energy-yielding substrate *in vivo* for experimental animals may be inferred from the report of Waelsch (1951) that the addition of glutamic acid to perfused cat brain caused a decrease in respiration. Maddock, Hawkins & Holmes (1939) found that glutamate, unlike glucose, was unable to abolish the changes in the electroencephalogram produced by the progressive hypoglycaemia of hepatectomized experimental animals. McIlwain (1951) showed that the addition of glutamate to respiring slices of guinea pig brain abolished the metabolic response to stimulation *in vitro*. However, the species studied is of some importance, for McIlwain (1953) reported that slices of human brain respiring with glutamate as substrate responded to *in vitro* stimulation. With the human tissue, glutamate did not abolish the usual response observed in the presence of glucose.

General. The possibility was considered that under the conditions where the ^{32}P incorporation was inhibited, non-labelled inorganic P might appear either in the slice or in the medium as a result of hydrolysis of P-containing compounds during the period of incubation. The effect of this would be to decrease the specific activity of the inorganic P of the medium and to increase the pool of inorganic P within the slice. McIlwain (1951; 1952*a*) and Gore & McIlwain (1952) reported that changes in the inorganic constituents of the medium, or the addition of L-glutamate, caused an increase in the concentration of inorganic P in the slice and McIlwain, Buchel & Cheshire (1951) showed that during the incubation of brain slices there was an increase in the concentration of inorganic P in the medium.

Table 8 shows the results of two experiments in which the concentration and the specific activity of the inorganic P in both the medium and the slice were determined before and after the 4 hr. incubation period. In each experiment there was a slight increase in the concentration of the inorganic P of the medium during the incubation period. The increase was slightly greater under conditions where the ^{32}P incorporation was inhibited. There was also an increase in the concentration of inorganic P in the slice during the incubation period. This increase tended to be less under the conditions where the ^{32}P incorporation was inhibited. These experiments are not directly comparable with those of McIlwain *et al.* (1951) and McIlwain (1952*a*), in that the incubation period was prolonged for 4 hr.

From Table 8 it can be seen that during the incubation there was, as to be expected, a decrease in the specific activity of the inorganic P of the medium and an increase in the specific activity of the inorganic P of the slice. The table also shows that under conditions where the ^{32}P incorporation was inhibited, there were only slight changes in the specific activity of the inorganic P of either the medium or the slice. This is well illustrated in Table 9, which shows the specific activities of the lipid P, PNA, ROP and PP for the two experiments reported in Table 8. Not only was the specific activity of each fraction decreased under conditions such as an increase in the concentration of K^+, or the presence of glutamine or NH_4^+, but the specific activity relative to that of the inorganic P of the slice was also decreased. These figures indicate that the inhibition of ^{32}P incorporation cannot be explained merely in terms of changes in the specific activity of inorganic P, either in the medium or in the slice.

The relative specific activity figures reported in Table 9 do not represent the turnover of the respective P-containing fractions, for part of the inorganic P of the slice is extracellular and part is intracellular. At present there is no satisfactory way of determining the specific activity of the intracellular portion of the inorganic P.

Table 9. *Specific activity and relative specific activity of lipid* P, *PNA, ROP and PP of slices of cat brain*

Slices incubated for 4 hr. at 37·2° in medium containing glucose (0·011 M) and [^{32}P]phosphate.

	Specific activity (counts/min./μg. P)				Specific activity relative to specific activity of inorganic P of slice × 100			
	Lipid P	PNA	ROP	PP	Lipid P	PNA	ROP	PP
Expt. 1								
No glutamine, normal K^+	12·1	26·1	162	540	0·40	0·86	5·3	17·8
Glutamine (0·01 M), normal K^+	8·8	16·3	93	400	0·33	0·60	3·4	14·9
No glutamine, K^+ (29 mM)	8·2	15·3	109	440	0·26	0·48	3·4	13·8
Expt. 2								
No NH_4^+	27·3	—	—	—	0·52	—	—	—
NH_4^+ (29 mM)	8·2	—	—	—	0·17	—	—	—

In previous papers attention has been drawn to the similarity that exists between the conditions that depress the incorporation of ^{32}P into the lipid P and PNA of cat brain slices and the conditions, studied by McIlwain and his colleagues, that bring about a decrease in the concentration of creatine phosphate, ATP, and possibly other labile phosphate in respiring slices of guinea pig brain (see McIlwain *et al.* 1951; McIlwain, 1952*a*, *b*; Kratzing & Narayanaswami, 1953). These conditions include the absence of oxygen or glucose, replacing the glucose by L-glutamate or succinate, and the presence of a large range of metabolic inhibitors, including 2:4-dinitrophenol (Findlay *et al.* 1953; Strickland, 1954). To these may now be added the addition of L-glutamate or L-glutamine to the medium, the removal of Ca^{2+}, the addition of NH_4^+, and an increase in the concentration of K^+.

SUMMARY

1. An increase in the concentration of K^+, omission of Ca^{2+}, or the addition of NH_4^+ to the incubating medium caused a decrease in the incorporation of [^{32}P]phosphate into the lipid P, pentosenucleic acid (PNA), residue organic P (ROP) and 'phosphoprotein' P (PP) of slices of cat brain respiring in a Krebs–Ringer bicarbonate buffer containing glucose and inorganic phosphate labelled with ^{32}P.

2. The addition of L-glutamate or L-glutamine to the medium, either in the presence or the absence of glucose, caused a decrease in the incorporation of ^{32}P into the lipid P, ROP and PP.

3. The inhibition of ^{32}P incorporation was not the result of changes in the specific activity of inorganic P, either in the medium or in the slice.

4. The similarity between the conditions that adversely affect the incorporation of ^{32}P into the P-containing fractions of brain slices and those that cause a failure of brain slices to maintain their concentration of phosphocreatine is commented upon.

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Chemical Studies of Peripheral Nerve during Wallerian Degeneration

6. INCORPORATION OF RADIOACTIVE PHOSPHATE INTO PENTOSENUCLEIC ACID AND PHOSPHOLIPIN *IN VITRO*

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In previous papers from this laboratory the changes that occur in the concentration of lipids (Johnson, McNabb & Rossiter, 1949; Burt, McNabb & Rossiter, 1950), nucleic acids (Logan, Mannell & Rossiter, 1952*b*), and certain enzymes (Hollinger, Rossiter & Upmalis, 1952; Hollinger & Rossiter, 1952) have been described for peripheral nerves degenerating after nerve section or regenerating after nerve crush. After nerve section there is a decrease in the concentration of the principal lipid components of the myelin sheath of the nerve fibre (myelin lipids) and a decrease in the concentration of the residue organic phosphorus (ROP), referred to as 'inositide P' by Logan, Mannell & Rossiter (1952*a*). There is also a smaller decrease in the concentration of 'phosphoprotein' phosphorus (PP) and an increase in the concentration of both deoxypentosenucleic acid (DNA) and pentosenucleic acid (PNA), associated with the cellular proliferation and the infiltration of macrophages that accompany the degeneration. If the nerve subsequently regenerates (after crush instead of section), the concentrations of myelin lipids, ROP and PP gradually return to normal and the concentrations of DNA and PNA decrease.

Since it has been shown that inorganic phosphate labelled with ^{32}P is incorporated into both the phospholipin (Fries, Schachner & Chaikoff, 1942; Schachner, Fries & Chaikoff, 1942; Strickland, 1954) and the PNA (DeLuca, Rossiter & Strickland, 1953; Findlay, Rossiter & Strickland, 1953) of brain slices respiring in a Krebs–Ringer bicarbonate medium containing glucose, it was decided to investigate the possible incorporation of ^{32}P into the lipids and PNA of normal, degenerating and regenerating peripheral nerve under similar conditions. In addition, observations were made on the incorporation of ^{32}P into the ROP and PP of normal, degenerating and regenerating nerve (see Strickland, 1952; Findlay, Strickland & Rossiter, 1954).

A preliminary report of some of these experiments has already appeared (Magee & Rossiter, 1954).

METHODS

The right sciatic nerve of a number of cats was either sectioned or crushed at the level of the greater trochanter of the femur. Details of the operations have been described previously (Johnson *et al.* 1949; Burt *et al.* 1950). No attempt was made to control the age, sex, or weight of the animals. After 8, 16, 32 or 96 days the animals were killed, and the segment of the nerve distal to the site of the operation was removed. At the same time a similar length of left sciatic nerve was removed to serve as a control. Each nerve was cleaned of adherent fatty and connective tissue, the epineurium was stripped off and the nerve was rapidly weighed. It was then cut into small lengths (about 1 mm.) and transferred to a Warburg flask containing Krebs–Ringer bicarbonate, glucose and [^{32}P]phosphate. The vessel was filled with a mixture of 95% O_2 and 5% CO_2 and incubated in a bath at 37·2° for 4 hr., with shaking to ensure proper oxygenation. Details of the incubation have been described by DeLuca *et al.* (1953).

After the incubation the nerve fragments were transferred to a mortar and the excess liquid was poured off. They were then frozen with liquid nitrogen and ground to a fine powder. This powder was added to the incubating fluid and mixed with 5 ml. cold 20% (w/v) trichloroacetic acid (TCA) to give a final concentration of 10% TCA. The tube and contents were kept at 0–4° by means of an ice bath. The acid-soluble P was removed as described by DeLuca *et al.* (1953). The residue was separated into lipid, PNA, ROP and PP and each fraction was purified as described by Findlay *et al.* (1954). The specific activity of the P in each purified fraction was then determined. The nucleic acids were extracted by the method of Hammarsten (1947), after which the PNA nucleotides were subjected to two magnesia treatments. For brain slices the mixed nucleotides so obtained have a specific activity within the range given by the individual nucleotides separated chromatographically (DeLuca *et al.* 1953). In experiments in which the nerves from a number of cats were pooled, the same was shown to be true for the nucleotides of the PNA of cat sciatic nerve.

All values were corrected for decay and calculated on the basis of a standard count of $8{\cdot}1 \times 10^5$ impulses/min./ml. incubating medium.

RESULTS

Normal nerves. Table 1 shows the specific activity of each of the four P-containing fractions of the normal sciatic nerve of the cat after incubation for 4 hr. in the medium containing glucose and [^{32}P]phosphate. In each experiment the right and the left sciatic nerve were incubated separately. The figures have not been subjected to any detailed statistical analysis, but it is evident that for each fraction the variation between animals is greater than the variation between the right nerve and the left nerve of the same animal. The table also shows that the specific activities of the P-containing fractions were in the order: PP > ROP > PNA > lipid P.

Table 2 shows the mean activity of each fraction expressed in terms of the number of impulses/min./100 mg. fresh nerve. The figures were calculated from the mean concentration of P in the fraction, as reported by Logan *et al.* (1952*b*), and the mean specific activity, as reported in Table 1. It can be seen that, although the specific activity of the PNA was considerably greater than that of the lipid P, the concentration was much less, so that per 100 mg. nerve, less ^{32}P was incorporated into the PNA than into the lipid. Also more ^{32}P was incorporated into the ROP, for this fraction had both a higher concentration and a higher specific activity than the PNA. Although the PP concentration was low, the specific activity of the fraction was very high, so that, in terms of fresh weight of nerve, the activity of the PP was of the same order as that given by the lipid P.

In Table 3 are reported the results of two experiments in which the specific activities were determined either before or after the incubation period. The 4 hr. values are for a nerve incubated in the usual way. The 0 hr. values are for the sciatic nerve from the opposite side of the body. This nerve was prepared as usual and placed in the vessel containing the medium and the [^{32}P]phosphate. The vessel was shaken to ensure thorough mixing and the nerve fragments were then removed and frozen. The proteins were precipitated with TCA in the presence of the [^{32}P]phosphate, as was customary for the in-

Table 1. *Specific activity of the phosphorus compounds of the sciatic nerve of the cat*

Nerve fragments incubated at 37·2° in Krebs–Ringer bicarbonate containing [^{32}P]phosphate. Glucose, 0·011 M. Incubation time, 4 hr. Counts/min./μg. P.

	Lipid P		PNA		ROP		PP	
Expt. no.	Right nerve	Left nerve	Right nerve	Left nerve	Right nerve	Left nerve	Right nerve	Left nerve
1	6·1	6·9	63	72	104	146	1070	1270
2	10·8	10·8	90	114	204	241	1030	1200
3	6·3	5·8	89	92	119	80	900	810
4	10·8	9·8	116	94	217	160	1600	1440
5	8·4	8·6	91	76	166	166	1890	1930
6	7·5	8·5	—	—	125	136	1470	1860
7	8·2	8·9	125	150	179	247	1230	1350
Mean	8·4		98		164		1360	

Table 2. *Activity of the phosphorus compounds of sciatic nerve of the cat*

	Concn. (μg. P/100 mg. wet tissue ± S.E.M. From data of Logan *et al.* 1952*b*)	Mean specific activity (counts/min./μg. P. From Table 1)	Activity (counts/min./100 mg. wet tissue)
Lipid P	310 ± 5·6	8·4	2600
PNA	3·9 ± 0·1	98	380
ROP	9·0 ± 0·4	164	1500
PP	1·8 ± 0·1	1360	2400

cubated nerves. The table shows that only small amounts of the ^{32}P were incorporated into the 0 hr. nerve. These findings indicate that the purification procedure for each of the fractions was satisfactory. They also suggest that the incorporation of ^{32}P into each of the fractions is a metabolic phenomenon.

Strickland (1954) and Findlay *et al.* (1953) reported similar results for the lipid P and PNA of brain slices. These workers also showed that the incorporation of ^{32}P was abolished by anaerobic conditions, by the addition of metabolic inhibitors, or by the addition of 2:4-dinitrophenol in concentrations that do not decrease the oxygen uptake. It thus seems likely that the incorporation of ^{32}P into each of the fractions is dependent upon the existence of oxidative phosphorylation processes within the nerve.

Sectioned nerves. Nerves degenerating for different periods of time after nerve section were incubated for 4 hr. in the medium containing glucose and [^{32}P]phosphate. Table 4 shows the change in the mean specific activity of each fraction. The specific activities of the degenerating nerves are expressed as percentages of the specific activities of the control nerves from the same animals.

After nerve section the specific activity of the lipid P increased with each successive period of degeneration, to reach a value greater than 12 times that of the control nerve by the end of 96 days. In contrast, the specific activity of the PNA decreased during the early stages of the degeneration, falling to 60 % of that of the control nerves by the end of 16 days. At the 32- and 96-day periods the specific activity of the PNA of the sectioned nerves did not differ significantly from that of the controls.

The specific activity of the ROP fraction, like that of the PNA, was less than normal at 8 and 16 days after the operation. The specific activity of this fraction then commenced to increase. By 32 days it did not differ significantly from that of the control nerves, and by 96 days it was significantly greater. The only time interval at which the specific activity of the PP was greater than that of the controls was at 96 days.

Table 3. *Specific activity of the phosphorus compounds of the sciatic nerve of the cat before and after incubation*

Nerve fragments incubated at 37·2° in Krebs–Ringer bicarbonate containing [^{32}P]phosphate. Glucose, 0·011 M. Counts/min./μg. P.

	Expt. 1		Expt. 2	
	0 hr.	4 hr.	0 hr.	4 hr.
Lipid P	0·5	8·4	0·4	7·7
PNA	2	113	2	148
ROP	3	178	5	203
PP	70	1130	57	1170

Table 4. *Effect of nerve section or nerve crush on the specific activity of the phosphorus compounds of the sciatic nerve of the cat*

Incubation conditions as in Table 1. Results are expressed as (specific activity degenerating nerve ÷ specific activity control nerve) × 100. No. of animal is stated in parentheses under each result. *P* gives the significance of the change reported.

Time after section (days)	Lipid P			PNA			ROP			PP		
	Mean	S.E.M.	*P*	Mean	S.E.M.	*P*	Mean	S.E.M.	*P*	Mean	S.E.M.	*P*
A. Nerve section												
8	132	± 15 (4)	<0·1	80	± 8 (4)	<0·05	54	± 5 (4)	<0·01	88	± 8 (4)	>0·3
16	226	± 24 (10)	<0·01	61	± 4 (6)	<0·01	55	± 8 (10)	<0·02	95	± 9 (9)	>0·4
32	678	± 106 (3)	<0·05	100	± 18 (3)	>0·9	104	± 40 (3)	>0·8	108	± 42 (3)	>0·7
96	1290	± 64 (7)	<0·001	121	± 20 (6)	>0·6	173	± 18 (7)	<0·01	156	± 22 (7)	<0·05
B. Nerve crush												
Time after crush (days)												
32	548	± 73 (4)	<0·02	104	± 15 (4)	>0·9	130	± 52 (3)	>0·7	84	± 28 (3)	>0·4
96	540	± 56 (6)	<0·001	112	± 18 (6)	>0·3	209	± 22 (6)	<0·05	165	± 23 (6)	>0·1

Table 5. *Effect of nerve section or nerve crush on the activity of the phosphorus compounds of the sciatic nerve of the cat*

Time after section (days)	Lipid P: Mean concn. (mg. P/100 g. wet tissue ±S.E.M.)	Lipid P: Mean specific activity (counts/min./ μg. P)	Lipid P: Activity (counts/min./ 100 mg. wet tissue)	PNA: Mean concn. (mg. P/100 g. wet tissue ±S.E.M.)	PNA: Mean specific activity (counts/min./ μg. P)	PNA: Activity (counts/min./ 100 mg. wet tissue)
			A. Nerve section			
0	310±5·6	8·4	2600	3·9±0·1	98	380
8	293±22	11·1	3250	14·8±0·7	79	1170
16	156±20	19·0	3000	18·5±0·8	60	1110
32	79±10	52	4100	20·8±2·5	98	2000
96	22±2	108	2400	9·8±0·8	119	1170
			B. Nerve crush			
Time after crush (days)						
32	78±12	46	3600	17·0±2·4	103	1750
96	110±9	45	4950	9·0±1·2	110	990

The effect of nerve section on the concentration of the four fractions under study was reported by Logan *et al.* (1952*b*). Table 5 shows the mean concentrations and the mean specific activities of the lipid P and PNA at each of the time intervals after nerve section. From these figures has been calculated the activity of each of the fractions in terms of the number of impulses/min./100 mg. wet nerve. The activity of the lipid P/100 mg. nerve increased with each successive time period after nerve section up to 32 days, and then decreased by 96 days. For the PNA, the activity/100 mg. nerve was greater than that of the control nerves at all time intervals after section. The activity/100 mg. reached a maximum at 32 days, at which time the concentration of PNA was at its greatest and the specific activity had returned to normal.

Crushed nerves. Table 4 also shows the results for nerves regenerating 32 or 96 days after nerve crush. At 32 days after the operation the specific activity of no fraction differed significantly from that of the corresponding fraction 32 days after nerve section. The specific activity of the lipid P was significantly greater than that of the lipid P of the control nerves, but the specific activity of each of the other fractions did not differ significantly from the values for the intact controls.

At 96 days the specific activity of the lipid P of the crushed nerves, although still much greater than that of the controls, was significantly less than that of the sectioned nerves. Table 5 shows that at this time interval considerable re-myelination had taken place, as demonstrated by the increase in the concentration of lipid P. At 32 days after nerve crush the figures for the activity of the lipid P/100 mg. nerve were not significantly different from those reported for nerve section, but by 96 days, the activity of the regenerating nerves had become more than twice that of the degenerating sectioned nerves.

The changes observed after nerve crush in both the specific activities and the activities/100 mg. nerve for the PNA, ROP and PP fractions did not differ significantly from the corresponding values reported after nerve section (Tables 4 and 5).

By 96 days after the operation both the concentrations and the specific activities of the lipid P and PNA were returning towards the values given by the intact nerves (Table 5). By this time axons from the undamaged portion of the nerve proximal to the site of injury had grown down into the peripheral portion, and re-myelination was proceeding. It is of interest to note that the activity of the lipid P/100 mg. nerve was higher 96 days after nerve crush than at any other period studied.

Are the results caused by a barrier limiting the inward diffusion of inorganic ^{32}P into the nerve?

Feng & Gerard (1930) found that for the sciatic nerve of the frog the connective tissue sheath surrounding the nerve may act as a diffusion barrier for certain inorganic ions. The experiments of Mullins & Grenell (1952) with frog nerve and Causey & Palmer (1953) with rabbit nerve demonstrated that the epineural sheath functions as a diffusion barrier for inorganic ^{32}P. In our experiments the epineurium and surrounding connective tissue were stripped off the nerve and the remainder of the nerve was then cut into small pieces approximately 1 mm. in length. Before the nerve was cut up in this way it would correspond to the stripped nerve of Causey & Palmer (1953).

From Table 5 it can be seen that in the degenerating nerves the specific activity of the lipid P increased, and the concentration of the lipid P decreased, with each successive time period after nerve section. Table 5 also shows that during the early stages of the degeneration the specific activity of the PNA fell and the concentration of PNA

increased. These observations raised the possibility that during the incubation period some diffusion barrier within the nerve might limit the inward diffusion of inorganic ^{32}P to a constant amount and that only this amount might be available for incorporation into the P-containing fractions. Other conditions remaining the same, the specific activity of each fraction would then be determined by the P concentration of that fraction, a reduction in the concentration of P of the fraction causing an increase in the specific activity, and vice versa.

An examination of the figures presented in Table 5 shows that the incorporation of ^{32}P into either the lipid P or the PNA is not the same for each time interval after nerve section, even on a nerve-weight basis. Also, the relative changes in the activities for PNA are quite different from those for lipid P. These observations would be difficult to explain solely in terms of the restricted penetration of ^{32}P. In addition, there are other observations inconsistent with such an explanation, i.e. the finding that the peak in the concentration of PNA was at 32 days, whereas the minimum in the specific activity was at 16 days, and the finding that both the concentration (Logan *et al.* 1952*b*) and the specific activity of the ROP fell during the early stages of the degeneration.

Despite the fact that precautions were taken to remove any diffusion barrier, by the stripping of the epineurium and the cutting of the nerve into short lengths, it was decided, as a further check, to investigate the incorporation of ^{32}P into finely divided homogenate preparations. Since cat sciatic nerve could not be homogenized satisfactorily, the sciatic nerve of the rat was used for these experiments. The specific activities were studied in a suitably 'reinforced' system, similar to that used by Strickland, McMurray & Rossiter (to be published) for rat brain.

The sciatic nerve of each of two rats was sectioned on one side. After 20 days both intact and degenerating nerves were removed and homogenized in a homogenizer of the Potter–Elvehjem type. The pooled normal and pooled degenerating nerves were incubated in a medium containing (in final concentrations) the following: glucose, 0·028 M; hexose diphosphate (K salt), 0·005 M; adenosine triphosphate (K salt), 0·007 M; coenzyme I, 0·001 M; cytochrome *c*, 0·00006 M; potassium fumarate, 0·0016 M; nicotinamide, 0·04 M; $MgCl_2$, 0·008 M; glycylglycine buffer (pH 7·4) 0·083 M; phosphate buffer (pH 7·4), 0·01 M. Sufficient [^{32}P]phosphate, made up in 0·1 ml. neutralized buffer, was added to make the specific activity of the inorganic P of the incubating medium the same as that in the cat-nerve experiments. The vessels were filled with oxygen and the homogenates were incubated, with shaking, for 1 hr. The specific activities of the lipid P and PNA were then determined as described previously.

Table 6. *Specific activity of the phosphorus compounds in a homogenate of the sciatic nerve of the rat*

Homogenate incubated as described in text. Counts/min./μg. P.

	Expt. 1		Expt. 2	
	Control	20 days after section	Control	20 days after section
Lipid P	0·7	3·3	0·5	2·1
PNA	4·9	1·7	—	—

Table 6 shows that with such a system the specific activity of the lipid P was much greater 20 days after nerve section, whereas the specific activity of the PNA was less. These results, which are similar to those reported in Table 4 for fragments of cat nerve, provide additional evidence that the changes in specific activity are metabolic, and that they are not due to a restricted penetration of the inorganic ^{32}P into the nerve.

DISCUSSION

The finding that ^{32}P was incorporated into the lipid P of cat sciatic nerve respiring in a glucose-containing medium *in vitro* agrees with the previous report of Fries *et al.* (1942) for dog nerve. The demonstration of such an incorporation suggests that the phospholipins of the myelin sheath may be formed *in situ* and neither taken up from the blood stream as such, nor formed in the body of the nerve cell and passed down to the myelin sheath of the nerve fibre.

The experiments also indicate that ^{32}P is incorporated into the lipid P, PNA, ROP and PP of peripheral nerve. They are thus complementary to the previous findings that ^{32}P is incorporated into the corresponding fractions of cat brain slices (DeLuca *et al.* 1953; Strickland, 1954; Findlay *et al.* 1954). It is of interest to note that with cat sciatic nerve the figures are of the same order as those found in previous experiments with cat brain slices for lipid P, ROP and PP, but that for PNA the sciatic nerve figures are considerably greater (Findlay *et al.* 1954).

Lipid phosphorus. The increase in the specific activity of the lipid P in the degenerating nerves is in accord with the findings of Bodian & Dziewiatkowski (1950) and Samuels, Boyarsky, Gerard, Libet & Brust (1951). These workers administered [^{32}P]phosphate to monkeys and guinea pigs and reported a greater *in vivo* incorporation of the ^{32}P into the lipid P of the sectioned nerves than into the lipid P of intact nerves. Although such experiments *in vivo* might be considered more physiological, their interpretation is complicated by the possibility that the ^{32}P incorporation into the degenerating nerve might be affected by changes in the blood flow and by changes in the permeability of the blood vessels supplying the nerve. The blood flow could be

modified by changes in both the number and in the degree of dilation of these vessels. In spite of these complicating factors, there was an increase in the specific activity of lipid P during Wallerian degeneration both *in vivo* and *in vitro*.

An interpretation of the results in terms of existing knowledge of the chemistry of Wallerian degeneration is difficult. At the time of myelin destruction, when the concentration of lipid P is decreasing, more ^{32}P is incorporated into the lipid during the *in vitro* incubation. Several explanations are possible:

(i) As Wallerian degeneration proceeds, the myelin sheath breaks up physically, enabling the phospholipins of the myelin sheath to come into closer proximity to the inorganic ^{32}P, or some labelled P-containing precursor. The finding that there was an increase in the specific activity of the lipid P of an homogenate of rat nerve is evidence against this explanation.

(ii) The enzymes that catalyse the exchange between inorganic ^{32}P and the P of the phospholipin are present in Schwann cells and macrophages. These cells become more numerous and more active as the nerve degenerates, and so the concentration of enzymes in the *in vitro* system increases, reaching a maximum in 32 days. At 96 days after nerve section, when the specific activity of the lipid P is still very high, the cellularity of the nerve has greatly decreased (Logan *et al.* 1952*b*), but the cells are still metabolically active, as is evidenced by the high specific activity of the PNA.

(iii) The phospholipin of the myelin sheath is metabolically inert in so far as the incorporation of ^{32}P is concerned, and the metabolically active lipids are in cells such as the Schwann cells and macrophages. These cells increase in number during the course of the degeneration and it may be non-myelin lipid in these cells, rather than the rapidly disappearing myelin lipid, that takes up the ^{32}P. It is possible that the ^{32}P is incorporated into the individual phospholipins at different rates. The recent reports of Dawson (1953) and Streicher (1953) on the specific activities of the individual phospholipins of brain homogenates are particularly important in this regard.

Protein-bound phosphorus. For technical reasons our PNA, ROP and PP fractions cannot be compared with the fractions studied by Bodian & Dziewiatkowski (1950) and Samuels *et al.* (1951) in their experiments *in vivo*. The 'residual P' of Bodian & Dziewiatkowski (1950) included PNA, DNA, PP and our ROP, and the 'nucleic acid' of Samuels *et al.* (1951) included PNA, DNA and a considerable portion of our ROP. Since each of these components has a greatly different specific activity, and since the components vary greatly in their relative concentrations during Wallerian degeneration (Logan *et al.* 1952*b*), the specific activity of these fractions would not be expected to bear any simple relation to the specific activity of the PNA, as reported here.

The specific activity of the PNA was significantly decreased in the degenerating nerve at both 8 and 16 days after nerve section (Table 4). However, at all time intervals after section the activity/100 mg. nerve was greater for the PNA (Table 5). During the degeneration there was an increase in the cellularity of the nerve, as judged by the concentration of PNA.

The ROP fraction is of some interest. The specific activity of this fraction was less than that of the control nerves at both 8 and 16 days after nerve section (Table 4). Logan *et al.* (1952*a*, *b*) have suggested that the phosphorus of this fraction may be derived, in a large measure, from the phosphorus of the inositol-containing lipid-protein complex described by Folch & LeBaron (1951), and thought by them to be part of the classical neurokeratin of Kühne & Chittenden (1889). That the concentration of this fraction decreases during Wallerian degeneration is evidence in favour of this view. However, Middleton (to be published) has shown that this fraction, like the concomitant P of liver tissue described by Davidson & Smellie (1952), is comprised of a number of highly active P-containing compounds.

Little is known of the origin of the PP. The specific activity of the PP fraction is very high (Table 1), but the concentration is quite low. By 96 days after nerve section the specific activity of this fraction was greater than that of the controls.

Regenerating nerves. At 32 days neither the specific activity nor the concentration of any of the P-containing fractions of nerves regenerating after nerve crush differed significantly from the corresponding figures for nerves degenerating after section. The only difference observed in the regenerating nerves was for the lipid P at 96 days after the operation. At this time there was a significant increase in the concentration of lipid P and a decrease in its specific activity. However, the activity of the lipid P/100 mg. nerve was significantly increased. This increase is probably related to the production of new myelin that is occurring at this time.

SUMMARY

1. Inorganic phosphate labelled with ^{32}P was found to be incorporated into the lipid P, pentose-nucleic acid (PNA), residue organic P (ROP) and 'phosphoprotein' (PP) of the sciatic nerve of the cat respiring in a Krebs–Ringer bicarbonate medium containing glucose and [^{32}P]phosphate.

2. The specific activities of the fractions were in the order: PP > ROP > PNA > lipid P.

3. In nerves degenerating after section and incubated under the same conditions, there was a large increase in the specific activity of the lipid P. During the early stages of the degeneration (8 and 16 days after nerve section) there was a decrease in the specific activity of the PNA and ROP. At 8, 16 and 32 days after nerve section the activity of the lipid P and PNA/100 mg. nerve was greater than the corresponding figure for control nerves.

4. In regenerating nerves 32 days after nerve crush, the specific activity of each of the four P-containing fractions did not differ significantly from that of the corresponding fraction 32 days after nerve section. By 96 days after the operation the specific activity of the lipid P was significantly less than that of the lipid P of nerves at the same time interval after section. The activity/100 mg. nerve was considerably greater.

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Actinomycete Metabolism: Origin of the Guanidine Groups in Streptomycin

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Despite the great interest that streptomycin has evoked in recent years, very little is known about the biochemical pathways leading to its production by *Streptomyces griseus*. Karow, Peck, Rosenblum & Woodbury (1952) have demonstrated the incorporation of the labelled carbon of [^{14}C]glucose into streptomycin. They did not, however, submit their product to chemical degradation, so that the distribution of the radioactivity between the various parts of the molecule is unknown. Nor is it clear from their results whether glucose functions as a direct precursor of the trisaccharide molecule or is first degraded into smaller molecular fragments.

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Our first points of attack have been the guanidine side chains of the streptomycin molecule and the results set out here show that the carbon atoms concerned are derived largely, if not entirely, from carbon dioxide. The mode of biosynthesis of the guanidine groups as a whole does not present as clear a picture, but there is an indication that arginine takes part in the process.

EXPERIMENTAL

Fermentation. The organism, *Streptomyces griseus, albus* mutant (Dulaney, Z38) is described in the patent covering its production (Dulaney, 1951). The development of a vegetative inoculum in a special meat medium and its use to inoculate a soybean medium are also described in example 1

of the same patent, which includes details of the broth media and the conditions of growth.

A clear synthetic medium ('Lumb's medium', Chesters & Robinson, 1951) was also used, particularly when mycelium was required free from medium constituents. In such instances, mycelium from 48 hr. cultures (1 vol. medium) was washed 3 times with 0·01 M potassium phosphate pH 7·0 (1 vol.), and resuspended for further experiments in a suitable buffer (1 vol. 0·01 M potassium phosphate, pH 7·0).

A special shake flask, the details of which are shown in Fig. 1, was constructed for the growth of cultures in the presence of $^{14}CO_2$. The gas was continuously circulated during each experiment by means of a small pump; other components of the circuit were a large gas reservoir and a simple apparatus for generating $^{14}CO_2$ from Ba $^{14}CO_3$. The culture flask was enclosed in a wire cage, screwed at its base to a rotary shaker and held in position by tightly packing the rest of the cage with cotton wool. At harvest, CO_2 was removed from the system by altering the circulation so as to absorb the gases in saturated aqueous $Ba(OH)_2$.

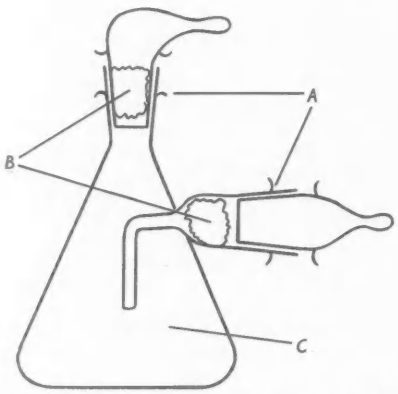
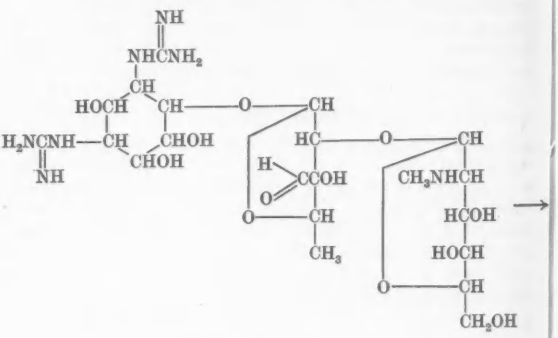


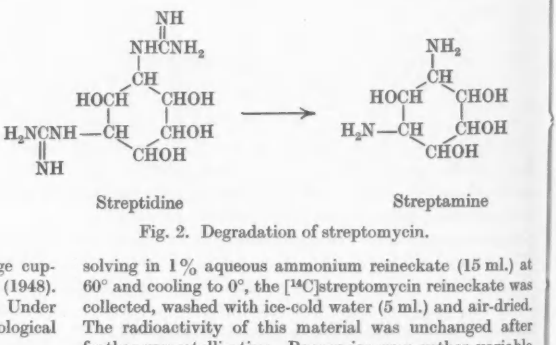
Fig. 1. Shake flask used for growing cultures in the presence of a circulated stream of gas. (*A*, ground glass cones and sockets; *B*, cotton-wool plugs; *C*, 250 ml. conical flask.)

was added to the combined filtrate and washings, which were then brought to neutrality by the addition of 40% (w/v) KOH. After further filtration if necessary, the volume of the solution was adjusted to 500 ml. with distilled water. The solution was percolated slowly (1 ml./min.) through a column containing 5 g. Amberlite IRC-50 cation-exchange resin (The Rohm and Haas Co., Philadelphia, Pa.) in the sodium cycle (Karow *et al.* 1952). All the streptomycin was retained when the solution was added at this rate. The resin was finally washed with water (50 ml.).

Elution was carried out very slowly with N-HCl (0·2 ml./min.); the initial neutral fraction (approx. 20 ml.) contained nearly all the [^{14}C]streptomycin. This fraction was immediately added to 2% aqueous ammonium reineckate (150 ml.) preheated to 65°. After cooling slowly to 30°, calcium reineckate was removed by filtration and the filtrate and aqueous washings (5 ml.) were allowed to stand at 0° for 24 hr. The resultant precipitate of [^{14}C]streptomycin reineckate was filtered off and washed with a small volume of ice-cold water. After recrystallization by dissolving in 1% aqueous ammonium reineckate (15 ml.) at 60° and cooling to 0°, the [^{14}C]streptomycin reineckate was collected, washed with ice-cold water (5 ml.) and air-dried. The radioactivity of this material was unchanged after further recrystallization. Recoveries were rather variable (60–90%, based on the bioassay of the original broth and of the added crystalline calcium chloride complex). A second crop of material of lower radioactivity was obtained from the combined mother liquors by co-crystallization with added unlabelled streptomycin reineckate. Conversion of the reineckate into sulphate was carried out, where necessary, by acidification of an aqueous solution with 2 N-H_2SO_4, and



Streptomycin



Streptidine Streptamine

Fig. 2. Degradation of streptomycin.

Streptomycin was assayed biologically by a large cup-plate method similar to that used by Brownlee *et al.* (1948). *Staphylococcus aureus* was used as the test organism. Under these conditions, 95–100% of the detected microbiological activity is accounted for by streptomycin.

Isolation of radioactive streptomycin. After removing a small portion for bioassay, the contents of the fermentation flask (40 ml.) were acidified to pH 2 with phosphoric acid. The flask was allowed to stand for 30 min. at room temperature, kieselguhr (2 g.) was added, and the mycelium was removed by filtration and washed well with water. About 1 g. of the pure calcium chloride complex of streptomycin

removal of Reinecke's acid by extraction into amyl acetate. Streptomycin sulphate was obtained from the aqueous phase by freeze-drying and crystallized from aqueous methanol.

Chemical degradation of streptomycin. The chemical structure of streptomycin and relevant degradation products are shown in the accompanying formulae (Fig. 2).

Streptomycin sulphate was dissolved in the minimum quantity of N-H_2SO_4 and allowed to stand for 1 week at room temperature. An equal volume of methanol was added and the solution was allowed to stand a further day when addition of 1 vol. of acetone completed the precipitation. After a few hours at 0°, the colourless rod-shaped crystals of streptidine sulphate were filtered off and recrystallized from a small volume of water.

Streptobiosamine dimethyl acetal was isolated from the mother liquors after removal of the sulphuric acid with Amberlite IR-4B (The Rohm and Haas Co., Philadelphia, Pa.) by evaporation *in vacuo* to remove the organic solvents and final freeze-drying. Although it proved to be unnecessary in the present work, this substance may be further degraded to L-*N*-methylglucosamine by acid hydrolysis (cf. Brink, Kuehl, Flynn & Folkers, 1946).

The streptidine sulphate was further degraded by boiling for 2 days with an excess of saturated aqueous $Ba(OH)_2$ in the absence of atmospheric CO_2. The mixture of barium salts (carbonate, sulphate and silicate from the glass of the apparatus) was then separated by centrifuging. The CO_2 was liberated from this by warming with 20% aqueous lactic acid and carried over in a CO_2-free air stream into saturated $Ba(OH)_2$. The resultant Ba $^{14}CO_3$ was again isolated by centrifuging, washed with water and acetone and dried in a vacuum oven at 50°. After separation of the insoluble barium salts, the excess of barium was removed from the original hydrolysate as $BaCO_3$ by addition of solid CO_2. Streptamine sulphate was then precipitated after acidification with sulphuric acid by addition of an equal volume of acetone. It was purified by crystallization from aqueous acetone.

Paper-chromatographic techniques. For the separation of compounds containing guanidine groups, the system used by Winsten & Eigen (1948) was modified. The solvent used for development contained *p*-toluenesulphonic acid (2%, w/v) dissolved in a mixture of water-saturated *n*-butanol (99 vol.) and piperidine (1 vol.). The same system was also used for the separation of α-amino acids. Other conventional systems that were found useful for this purpose included phenol–water (4:1, v/v) and lutidine–collidine–water (1:1:2, v/v). For the detection on paper of compounds containing guanidine groups, the diacetyl spray (Halliday, 1952) as modified by Foster & Ashton (1953) was used, and a freshly prepared 0·1% ninhydrin solution in ethanol for amino acids. All chromatograms were run on Whatman no. 4 paper.

Assay of ^{14}C. All radioactivity measurements were done on 2 cm.^{2} Polythene disks with solid samples of 'infinite thickness' (Popják, 1950).

RESULTS AND DISCUSSION

Incorporation of $^{14}CO_2$ *into streptomycin*

After 2 days' growth of *Streptomyces griseus* on the soybean medium (40 ml.), $^{14}CO_2$ was liberated from barium [^{14}C]carbonate (16 mg., containing 85 μc) and circulated continuously over the fermenting culture for 5 more days. Unreacted CO_2 was then trapped as barium carbonate (8·70 g., specific activity 1·75 μc/m-mole). Bioassay of a small portion revealed that the amount of streptomycin present in the broth was 75·0 ± 5·2 mg. Extraction as previously described after addition of unlabelled streptomycin calcium chloride complex containing 795 ± 20 mg. of streptomycin (bioassay) as carrier gave 1·70 g. of recrystallized streptomycin reineckate as the main crop. Assay for radioactivity showed, after making allowance for the dilution with unlabelled material, that the original undiluted streptomycin had a specific activity of 2·91 μc/m-mole. This represents an incorporation of between 0·4 and 0·5% of the $^{14}CO_2$ into streptomycin.

Further degradation, after conversion into streptomycin sulphate, showed that all the radioactivity was concentrated in the streptidine portion of the molecule. The streptidine sulphate was further diluted with unlabelled material before conversion into streptamine sulphate and barium carbonate. It was found that practically all the radioactivity of the streptidine was due to the carbon atoms of the guanidine side chains. The small amount found in the streptamine sulphate may have been due to contamination with a small quantity of unhydrolysed strepturea. The results are shown in Table 1.

It can be seen that the specific activity (1·56 μc/m-mole) of the barium carbonate obtained by degradation (and hence of the guanidine carbons) approaches very closely to the specific activity of the barium carbonate derived from the air circulating at the end of the original fermentation (1·75 μc/m-mole). The errors involved in the determination of these figures—bioassay determinations, measurement of broth volumes and radiochemical assays—are considerable, however, and the difference may not be significant. At first sight, it appears that the carbons of the guanidine side chains are entirely derived from CO_2, but, if these carbon atoms are not in complete equilibrium with the carbon dioxide in the circulating gas, the rates of $^{14}CO_2$ dilution and streptomycin synthesis have to be considered. As the circulating CO_2 is largely derived

Table 1. *Radioactivity of streptomycin degradation products*

Details of the production of streptomycin in the presence of $^{14}CO_2$ and of the chemical degradation are given in the Experimental section.

Substance	Specific activity (μc/m-mole)
Streptomycin sulphate	2·91
Streptidine sulphate	3·06
Streptamine sulphate	0·028
Barium carbonate (derived from guanidine side chains)	1·56

from the reducing sugars in the medium, the figures given in Table 2 are of interest in this connexion. In this typical serial experiment, flasks were removed each day, and assayed for reducing sugars (Shaffer & Hartmann, 1920) and streptomycin content. It can be seen that much of the reducing sugar had already been utilized, and hence the amount of CO_2 would remain relatively constant while most of the streptomycin was being produced by the organism.

To gain further information on this point, a second experiment with $^{14}CO_2$ was carried out under exactly the same conditions as the first one, except that growth was allowed to proceed for only 5 days altogether. The $^{14}CO_2$ was of the same initial specific activity as before. The results of the two experiments are compared in Table 3. The weight of the recovered barium carbonate in the second experiment approaches that obtained in the first, confirming that the rate of dilution of the $^{14}CO_2$ is at its greatest much earlier than the time of maximum streptomycin accretion. This would seem to indicate that the carbons of the guanidine side chains of streptomycin are, in fact, very largely derived from CO_2, but the lower degree of incorporation of radioactivity in the second (5-day) experiment poses a problem. Presumably, a proportion of the streptomycin, or, more probably, streptomycin precursors is elaborated by the organism during the first two days of growth. In the first experiment, where the culture was allowed to grow for seven days, the relatively large streptomycin production during the last two or three days of the fermentation would tend to obscure this effect.

Effect of delaying the passage of $^{14}CO_2$ on the extent of the incorporation of ^{14}C into streptomycin. In view of the above results, an attempt was made to determine whether further variation in the period during which $^{14}CO_2$ was supplied would result in any alteration in the extent of incorporation of ^{14}C into streptomycin. Three fermentations were carried out in which the passage of $^{14}CO_2$ was begun 2, 3 and 4 days after inoculation.

Streptomycin was isolated as usual after a total of 7 days' growth and the results are summarized in Table 4.

It can be seen that, when passage of $^{14}CO_2$ was begun 3 days after inoculation, the incorporation of ^{14}C into streptomycin was little different from that observed in the first experiment. However, if passage of $^{14}CO_2$ was delayed for 4 days, the labelling of the streptomycin was considerably lowered. The depression was greater than that to be expected if the effect were due solely to the streptomycin already synthesized, and we were again led to infer the presence of some intermediate acting as a depot for CO_2 and not in complete equilibrium with the gaseous phase. In view of the known role of arginine in transamidinations in biological systems, we decided to investigate the effect of this amino acid on the incorporation of $^{14}CO_2$.

Effect of arginine on the incorporation of $^{14}CO_2$. In this experiment, passage of $^{14}CO_2$ through two flasks linked in series was begun 2 days after inoculation. To one of the flasks L-arginine hydrochloride (200 mg.) dissolved in water (1 ml.) was added at the same time and to the control flask 1 ml. of water only was added. At harvest, the streptomycin from each flask was isolated after reserving a small portion for bioassay. A very marked difference in the incorporation of ^{14}C was observed in the two cases. The specific activity of the streptomycin isolated from the flask to which the arginine had been added was 0·401 μc/m-mole, whereas the product from the control culture had a specific activity of 0·627 μc/m-mole. There was little difference in the amounts of streptomycin produced in each flask (28 and 33 mg.). If one assumes that the carbons of the guanidine side chains are almost entirely derived from CO_2 in a normal fermentation, it is difficult to see how the arginine could depress the incorporation of ^{14}C in this experiment except by furnishing carbon atoms to replace those normally supplied by the gas. In this experiment, the unlabelled carbon atoms

Table 2. *Content of reducing sugar and streptomycin in the soybean medium during growth of* Streptomyces griseus

Length of fermentation (days)	Reducing sugars (as glucose) (mg./ml.)	Streptomycin content (μg./ml.)
0	25·9	—
1	25·1	—
2	15·4	—
3	10·8	250
4	7·3	511
5	2·5	923
6	1·5	1306
7	1·25	1461
8	1·25	1257

Table 3. *Incorporation of $^{14}CO_2$ into streptomycin*

Length of fermentation (days)	Initial wt. of $Ba^{14}CO_3$ (mg.)	Final wt. of $Ba^{14}CO_3$ (g.)	Final radioactivity of $Ba^{14}CO_3$ (μc/m-mole) A_1	Final radioactivity of streptomycin (μc/m-mole) A_2	A_2/A_1
7	16	8·70	1·75	2·91	1·7
5	30	7·30	3·19	3·16	0·99

present in the guanidine groups of the added arginine could have been used first for the synthesis of streptomycin, causing an apparent decrease in the extent of CO_2 fixation into streptomycin. Thus, these results suggest that arginine functions as an intermediate in the biosynthesis of streptomycin by a transfer of guanidine groups ('transamidination').

Experiments with washed mycelial suspensions

In these experiments, a study was made of the synthesis of substituted guanidines by the organism. Substrates were added at a level of 1 mg./ml. to the washed mycelial suspensions (40 ml., dry wt. of mycelium approx. 10 mg.) which were then incubated at 28° on a rotary shaker for 18 hr.

No conversion of streptamine (either hydrochloride or sulphate) into streptidine could be detected when washed suspensions were used under our conditions, whether arginine was present or not. However, by using the *p*-toluenesulphonic acid system and the diacetyl spray, a new unidentified compound containing at least one guanidine group was always detected by downward-flow paper chromatography. Methanol extracts of freeze-dried filtrates were used for the application of the spots to the papers.

Substituted guanidines all move slowly in this system, and it was only possible to obtain relative *R* values for them. These are shown in Table 5. Further investigations revealed that this same guanido-compound (GC) could also be obtained by incubating mycelial suspensions with streptidine hydrochloride or sulphate, so that streptamine is possibly converted first into streptidine by the mould and then rapidly into GC. We then found that GC could be obtained in the best yield from L-arginine (judging yield merely by the colour intensity of the diacetyl spot). It was also formed from creatine, creatinine, glycocyamine, guanidine, citrulline or ornithine. The function of this compound is not clear, but it may be involved in the biosynthesis of streptomycin.

GC was not detectable on spraying papers with the ninhydrin reagent under our conditions. Material containing GC and arginine, obtained from methanol extracts of freeze-dried filtrates, was subjected to alkaline hydrolysis by boiling with saturated aqueous barium hydroxide for 24 hr. On developing a methanol extract of the hydrolysate (after neutralization with sulphuric acid and freeze-drying), ninhydrin revealed the presence of ornithine and a further substance, presumably derived from GC. Using downward flow paper chromatography with phenol–water (4:1, v/v), this new compound had an *R* value of 1·39 relative to ornithine.

Table 4. *Effect of varying the time of* $^{14}CO_2$ *passage on the incorporation of* ^{14}C *into streptomycin*

For details of fermentations see text.

Time of $^{14}CO_2$ addition after inoculation (days)	Specific radioactivity of recovered $BaCO_3$ (μc/m-mole) A_1	Specific radioactivity of streptomycin (μc/m-mole) A_2	A_2/A_1
2	1·75	2·91	1·7
3	4·17	6·75	1·6
4	0·69	0·53	0·77

Table 5. *Comparative R values of substituted guanidines and α-amino acids in the* p*-toluenesulphonic acid system*

For details of the solvent system used see Experimental section.

Substance	R_A (relative to arginine)
L-Arginine	1·00
GC	1·43
Streptidine	3·45
Streptomycin	4·97
Mannosidostreptomycin	2·24
Creatine	0·87
Creatinine	0·96
Glycocyamine	0·81
Guanidine	3·50
L-Alanine	1·91
L-Lysine	0·66
L-Proline	1·16
L-Aspartic acid	0·35
L-Citrulline	0·45
DL-Ornithine	0·65, 0·69 (2 spots)

SUMMARY

1. Investigations carried out on *Streptomyces griseus* with the aid of $^{14}CO_2$ have shown that the carbons of the guanidine side chains in streptomycin are derived very largely, and possibly entirely, from carbon dioxide.

2. The maximum incorporation of $^{14}CO_2$ into streptomycin obtained was between 0·4 and 0·5 %. A much lower degree of fixation of ^{14}C was obtained when no $^{14}CO_2$ was passed for the first 72 hr. of a fermentation.

3. L-Arginine possibly functions as an intermediate in the biosynthesis of the guanidine side chains of streptomycin.

4. A variety of compounds, either containing guanidine groups or readily convertible into such compounds, are converted by *Streptomyces griseus* into a further substance containing at least one guanidine group. This compound has not yet been identified, but may be involved in the biosynthesis of streptomycin by the organism.

The authors acknowledge with pleasure the skilled technical assistance of Mrs F. Goodinson.

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Reduction of Dehydroascorbic Acid by Bacteria

3. ENZYMIC ACTIVATION OF DEHYDROASCORBIC ACID

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In the previous communications in this series (Eddy, 1952; Eddy, Ingram & Mapson, 1952) it has been shown that the mechanism by which *Escherichia coli* reduces dehydroascorbic acid (DHA) to ascorbic acid (AA) includes any of various dehydrogenases possessed by the organism and cytochrome b_1, and it has been suggested that there may be a further enzyme which acts on DHA. Because cell-free extracts of *Esch. coli*, however prepared, would not reduce DHA, this hypothetical activating enzyme could not be studied directly, but there remained several lines of attack which could strengthen the evidence for its existence. These were the use of leuco-Nile blue as the immediate hydrogen donor (Tarr, 1940; Jebb, 1949); the determination of the specific chemical configurations which are capable of combining with the enzyme; the investigation of the chemical specificity and kinetic behaviour of inhibitory substances, and experiments to find typical Michaelis-Menten relationships between the concentrations and rates of reduction of DHA and related reducible substances.

METHODS AND MATERIALS

Methods

The organisms used for this part of the work were two strains of *Esch. coli*: a reducing strain isolated in this laboratory and a strain (NCTC 7277) which we shall denote as the non-reducing strain, although in fact it does reduce DHA very slowly. The cells were grown and harvested as described previously (Eddy, 1952).

Thunberg tube experiments with Nile blue were carried out as follows. Into the body of the Thunberg tubes were introduced 3·2 ml. of 0·2 M phosphate buffer (pH 6·2) made from Na_2HPO_4 and KH_2PO_4, 0·1 ml. of M lithium lactate (to give the optimum concentration—Eddy, 1952, Fig. 2), 0·2 ml. of 0·2% (w/v) aqueous Nile blue sulphate (British Drug Houses Ltd.) and 1 ml. of bacterial suspension. A solution of 2 mg. DHA in 0·5 ml. of water was placed in the hollow stopper. The tubes were evacuated and placed in a water bath at 35°. When the dye had become reduced the DHA was added from the stopper and the oxidation of the dye measured by visual comparison with a series of colour standards. In a parallel series of tubes, serial AA estimations were carried out by opening the tube, adding 5 ml. 20% (w/v) metaphosphoric acid, diluting to a suitable volume and filtering. Samples were then titrated with 2:6-dichlorophenol indophenol.

Measurements of electrode potential were made in a 50 ml. beaker which was fitted with a rubber bung carrying a platinum electrode, a mercury–calomel half-cell and gas inlet and outlet tubes. It was immersed in a water bath at 35°. Into the beaker were put 17·5 ml. 0·2 M phosphate buffer (pH 6·2), 5 ml. of bacterial suspension and 0·5 ml. of M glucose. Air was removed by bubbling N_2 through the suspension. When the potential had reached a steady state after about 20–25 min., 2 ml. of DHA solution (4 mg./ml.) were added, and the ensuing potential changes measured. From a duplicate system samples were removed at intervals for AA determinations.

Experiments on the effect of inhibitors and H-acceptor concentration on the rate of reduction were carried out as described by Eddy (1952), the method involving serial titrations of AA on samples withdrawn from a reaction mixture containing H-donor, bacterial cells, DHA and buffer at pH 6·2, held at 35° under anaerobic conditions.

Materials

Of the compounds cited in Fig. 4, L-ascorbic acid, dihydroxymaleic acid, glyoxal, diacetyl, pyruvic acid and dihydroxytartaric acid (the hydrate of dioxosuccinic acid) were available commercially, as was also D-araboascorbic acid. The hydroxytetronic and reductic acids and the 3-hydroxy-1:2-dioxo-3-phenylbutyric acid lactone were given

by Professor T. Reichstein and Professor H. Dahl (Institute of Organic Chemistry, Basle University). The L-galactonic, L-gulonic and D-mannonic acid lactones and the D-galacturonic acid methyl ester were supplied by Dr F. A. Isherwood (for methods of preparation see Isherwood, Chen & Mapson, 1954). Butyrolactone was prepared by heating silver glutarate with I_2 (Sircar, 1928); dioxo-L-gulonic acid (as its Ca salt) by the first method of Penney & Zilva (1945), and methyl D-ribofuranoside by the HCl-catalysed methylation of D-ribose (Barker, 1948).

The unstable dehydro-compounds were prepared immediately before use by dissolving the enediols in glass-distilled water, adding Br_2 in slight excess and shaking. The remaining Br_2 was removed with a stream of air bubbled through the solution.

RESULTS

Chemical reaction between leuco-Nile blue and DHA. Before carrying out experiments similar to those of Tarr (1940) and Jebb (1949) it was desirable to find out whether there was a chemical reaction between reduced Nile blue and DHA. This was done as follows. The dye was reduced with bacteria under a layer of soft wax in centrifuge tubes, the cells were centrifuged down and DHA solution (previously deoxygenated by evacuation) was injected through the wax with a hypodermic syringe. A gradual restoration of colour took place showing that there is a chemical reaction; however, the rate was much less than that between oxygen and reduced Nile blue.

The rates of reaction in the presence of living and dead cells of the DHA-reducing strain of *Esch. coli* were next compared. The dye was reduced by a bacterial suspension in an atmosphere of nitrogen in a Thunberg tube and the cells were killed by heat before adding DHA solution from the stopper. The rate of oxidation of the dye was considerably lower than in unheated controls, but the full intensity of the colour corresponding to complete re-oxidation was not attained (Fig. 1). This was probably because some of the dye became adsorbed on the cells. The large difference between the two rates of oxidation of the leuco-dye is taken as evidence that there is an enzyme-catalysed reaction between DHA and reduced Nile blue in addition to a slower direct reaction.

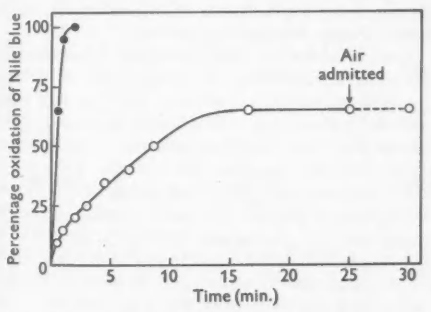


Fig. 1. Oxidation of leuco-Nile blue by dehydroascorbic acid. Experiments carried out in Thunberg tubes. Each tube contained 3·2 ml. 0·2 M phosphate buffer pH 6·2; 0·1 ml. M lithium lactate; 1·0 ml. bacterial suspension and 0·2 ml. 0·2% (w/v) Nile blue. Hollow stopper contained 2 mg. dehydroascorbic acid in 0·5 ml. water; temp. 35°. When dye reduced one tube heated at 100° for 2 min. to inactivate bacteria and dehydroascorbic acid then added. ●—●, control (unheated); ○—○, cells heated at 100° for 2 min.

Quantitative relationship between dye oxidation and DHA reduction. Previous workers (Jebb, 1949) found that oxidation of leuco-Nile blue by a hydrogen-acceptor was followed, after a lapse of time, by reduction. DHA behaved similarly in these tests. With glucose-grown cells the dye remained at its maximum level of oxidation for a period and was then quickly reduced, whereas with lactate-grown cells the dye attained a lower maximum level of oxidation and almost immediately began to be slowly reduced. The experiments were therefore repeated and the oxidation of leuco-Nile blue in Thunberg tubes observed simultaneously with measurements of the quantity of AA produced in duplicate tubes.

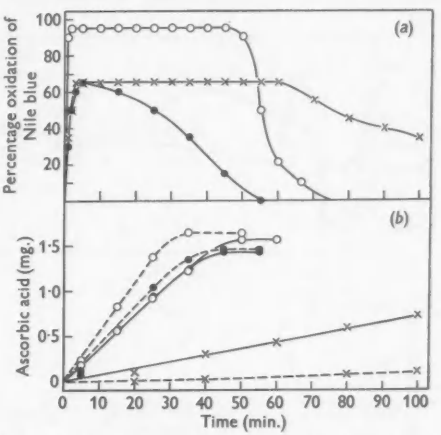


Fig. 2. (*a*) The effect of *Esch. coli* on oxidation of leuco-Nile blue by dehydroascorbic acid. (*b*) Reduction of dehydroascorbic acid in presence and absence of leuco-Nile blue. Experimental conditions as for Fig. 1 with lactate as H-donor and ascorbic acid concn. $2·3 \times 10^{-3}$ M. ○—○, reducing strain, glucose-grown, Nile blue present; ○- - -○, reducing strain, glucose-grown, Nile blue absent; ●—●, reducing strain, lactate-grown, Nile blue present; ●- - -●, reducing strain, lactate-grown, Nile blue absent; ×—×, non-reducing strain, glucose-grown, Nile blue present; ×- - - -×, non-reducing strain, glucose-grown, Nile blue absent.

Cells of the reducing strain, grown on either lactate or glucose, and of the non-reducing strain grown on glucose, were tested and the results, which were reproducible, are shown in Fig. 2. Two features are of interest. The first is that although in the presence of leuco-Nile blue both glucose-grown and lactate-grown cells reduce DHA at the same rate, with the latter, Nile blue so formed becomes reduced almost immediately, whereas with the former it does not become reduced until all the DHA has been reduced. The second is that whereas Nile blue diminishes the rate of reduction of DHA by glucose-grown and lactate-grown cells of the reducing strain it increases the rate by the non-reducing strain. This is presumably due to the chemical reaction between DHA and reduced Nile blue.

Measurements of electrode potential. Green, Stickland & Tarr (1934) emphasized that leuco-Nile blue does not necessarily play a major part in reducing the hydrogen acceptor under examination; but rather its function is to show whether addition of the hydrogen acceptor to the bacterial suspension raises the overall oxidation–reduction potential. However, in the present experiments (see Fig. 2), the 95% 'plateau' of the curve for the glucose-grown reducing strain suggested that the potential attained in this system extends well beyond the range for which Nile blue is useful. For this reason, and also because Nile blue appeared to be inhibiting the reduction of DHA, the Nile blue was omitted and the oxidation–reduction potential followed electrometrically. The AA produced was measured simultaneously on samples withdrawn from a duplicate system. The results (Fig. 3) show that the maximum E_H reached by the glucose-grown cells after the addition of DHA (+20 mv, an increase of 260 mv) was attained in 5 min., after which it began to fall. With lactate-grown cells, the maximum potential reached was 20 mv lower, i.e. 0 mv (an increase of 150 mv) and was attained in 25 min. As might be expected from the results with Nile blue, the potential with the non-reducing strain rose in a similar fashion to that with lactate-grown reducing strain but, within the duration of the experiment, showed little sign of attaining its maximum value, still less of declining.

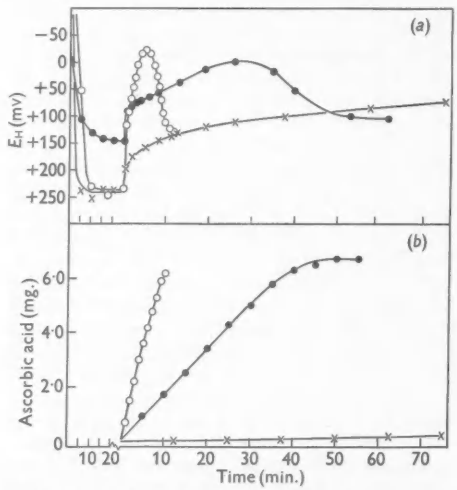


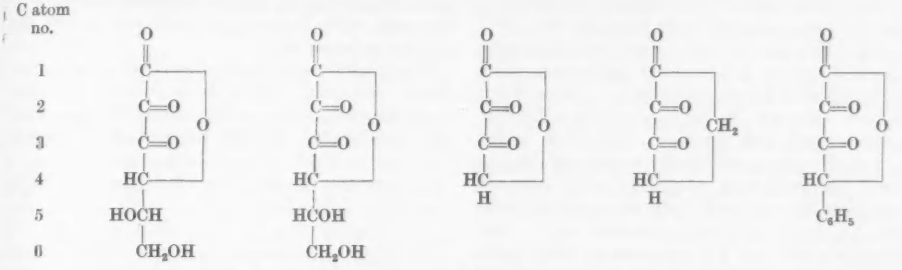
Fig. 3. (*a*) Effect of dehydroascorbic acid (initial concn. $1{\cdot}84 \times 10^{-3}$M) on the steady-state oxidation–reduction potential attained by washed suspensions of *Esch. coli* +0·02M glucose +0·14M phosphate buffer (pH 6·2) under N_2 at 35°. (*b*) Reduction of DHA under identical conditions. ○—○, reducing strain glucose-grown; ●—●, reducing strain lactate-grown; ×—×, non-reducing strain glucose-grown.

The specificity of the reduction. Earlier observations (Mapson & Ingram, 1951) showed that besides DHA, the oxidized forms of D-araboascorbic (D-*iso*ascorbic), hydroxytetronic and reductic acids, are also reducible. These observations have been confirmed and extended by showing that 3-hydroxy-1:2-dioxo-3-phenylbutyrolactone is also reduced.

Because the three last compounds lack carbon atoms 5 and 6 of DHA (see Fig. 4*a*), the remainder of the molecule has been investigated. In trials with diacetyl and dihydroxytartaric acid (the hydrated form of dioxosuccinic acid) the latter was not reduced; and results with the former were inconclusive because the compound produced by a reduction analogous to that of DHA would be the enol form of acetoin, which would be immediately converted into the keto form. The detection of acetoin, however, involves initial oxidation to diacetyl (Voges–Proskauer reaction).

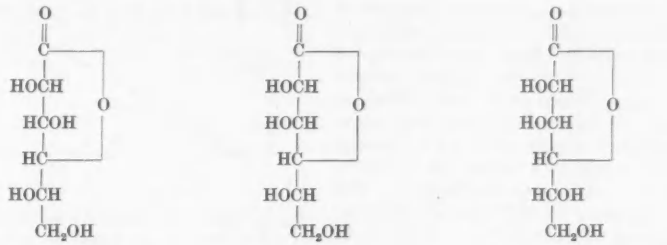
Specific inhibition of the reduction. As a potential competitive inhibitor, a compound of related chemical structure is needed which is not itself reduced by the system. It is desirable, too, to test it against the substrate at a substrate concentration which does not saturate the system: $3{\cdot}5 \times 10^{-4}$M DHA is appropriate, as will appear later (cf. Fig. 8). Ascorbic acid was an obvious possibility, since the products of enzyme action often combine with the enzyme; but the constant rate of reduction of DHA at adequate concentrations (cf. Mapson & Ingram, 1951), and the fact that the equilibrium of the reaction is so greatly in favour of AA that the reaction appears to be irreversible, suggest that AA does not interfere. Observations made to confirm this with higher ratios of AA:DHA were not accurate because the presence of much AA gives a large blank in the titrations which measure the reduction of DHA, but it was quite definite that there was no inhibition with 4 times as much AA as DHA. Dihydroxymaleic acid behaved similarly.

(*a*) Compounds reduced by *Esch. coli*



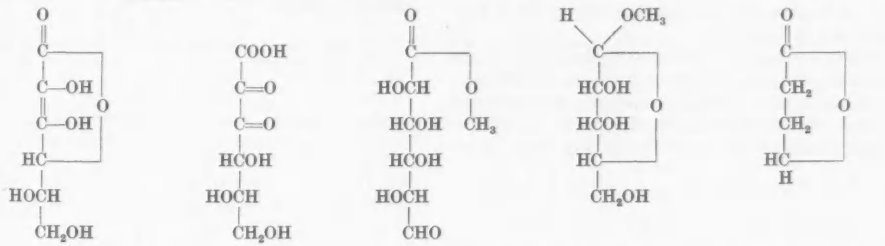
Dehydro-L-ascorbic acid | Dehydro-D-arabo-ascorbic acid | Dehydrohydroxy-tetronic acid | Dehydroreductic acid | 3-Hydroxy-1:2-dioxo-3-phenyl-butyro-γ-lactone

(*b*) Compounds inhibiting the reduction of DHA by *Esch. coli*

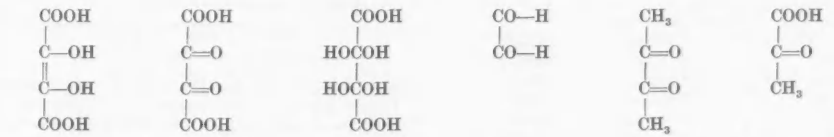


L-Galactono-γ-lactone | L-Gulono-γ-lactone | D-Mannono-γ-lactone

(*c*) Compounds neither reduced, nor inhibiting the reduction of DHA, by *Esch. coli*



L-Ascorbic acid | Dioxo-L-gulonic acid | D-Galacturonic acid methyl ester | Methyl D-ribo-furanoside | γ-Butyrolactone



Dihydroxy-maleic acid | Dioxosuccinic acid | Dihydroxytartaric acid | Glyoxal | Diacetyl | Pyruvic acid

Fig. 4.

These observations were consistent with the view that the critical centre of the molecule is the 1:2-diketo grouping, and that compounds with this grouping might inhibit. The most promising substance seemed to be oxidized reductone (*enol* tartronaldehyde) because this is not reduced by *Esch. coli* (Mapson & Ingram, 1951). Further investigation showed, however, that after oxidation with bromine, reductone could not be regenerated by hydrogen sulphide, and it seemed certain that the oxidized form is completely unstable. Glyoxal, diacetyl, pyruvic acid, 2:3-dioxogulonic acid and dihydroxytartaric acid were also tested and, at 10 times the molarity of the DHA (i.e. at $3{\cdot}5 \times 10^{-3}$ M) none of them diminished the rate of reduction of DHA by *Esch. coli*.

It seemed probable therefore that the ring structure was necessary and, since no further 5-membered ring compounds incorporating diketo groupings were available, we tried the γ-lactones of L-gulonic, L-galactonic and D-mannonic acids (see Fig. 4*b*). All of these inhibited the reduction of DHA (Fig. 5), in greater or lesser degree, without affecting the rate of methylene blue reduction under comparable conditions. The results of more detailed tests with $1{\cdot}75 \times 10^{-3}$ M; $3{\cdot}5 \times 10^{-4}$ M and $1{\cdot}2 \times 10^{-4}$ M DHA, and $3{\cdot}5 \times 10^{-3}$ M and 7×10^{-3} M L-gulono-γ-lactone are shown in Table 1. The activities are expressed as $Q_{H_2}^{DHA}$ (μl. H_2 at s.t.p. transferred/mg. dry wt. bacteria/hr.). In Fig. 6 the reciprocals of these values are plotted against the reciprocals of the respective DHA concentrations. It is clear that the inhibition is of the competitive type, since the maximum rate of reduction is unaffected but the K_m increases as the inhibitor concentration is raised.

As these compounds differ at carbon atom 5, this confirmed that the grouping there is of secondary importance. The necessity for the ring structure was verified when it was found that the methyl ester of D-galacturonic acid had no inhibitory effect, since this compound (in the straight chain form which exists in the equilibrium mixture) is otherwise identical with L-galactono-γ-lactone at the four critical carbon atoms.

The inhibition by these γ-lactones shows that the diketo grouping cannot be essential, but since butyrolactone, at a molar concentration found effective for the other inhibitors, had no effect on

Table 1. *Inhibition of the reduction of dehydroascorbic acid (DHA) by* L-*gulono-*γ*-lactone*

Experiments carried out at 35° under N_2 with 0·02 M glucose as H-donor in phosphate buffer pH 6·2. Results expressed as $Q_{H_2}^{DHA} = \mu$l. H_2 at s.t.p. transferred/mg. dry wt. bacteria/hr.

Concn. of DHA ($M \times 10^4$)	Concn. of lactone ($M \times 10^3$)	$Q_{H_2}^{DHA}$
17·5	0	45·5
17·5	3·5	34·0
17·5	7·0	25·0
3·5	0	42·0
3·5	3·5	14·5
3·5	7·0	9·0
1·2	0	29·0
1·2	3·5	5·5
1·2	7·0	3·5

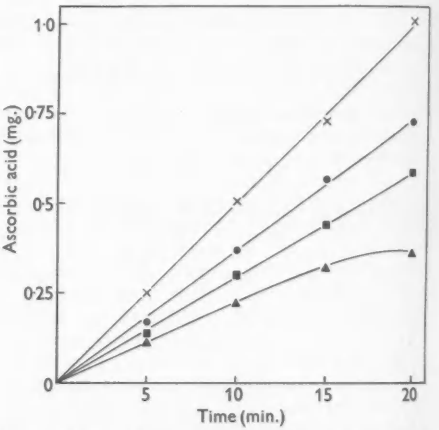


Fig. 5. Effect of aldonic acid γ-lactones ($3{\cdot}5 \times 10^{-3}$ M) on the rate of reduction of dehydroascorbic acid (initial concn. $3{\cdot}5 \times 10^{-4}$ M) by washed suspensions of *Esch. coli* using glucose as H-donor at 35°; with 0·15 M phosphate buffer pH 6·2. ×—×, control, no lactone; ●—●, D-mannono-γ-lactone; ■—■, L-galactono-γ-lactone; ▲—▲, L-gulono-γ-lactone.

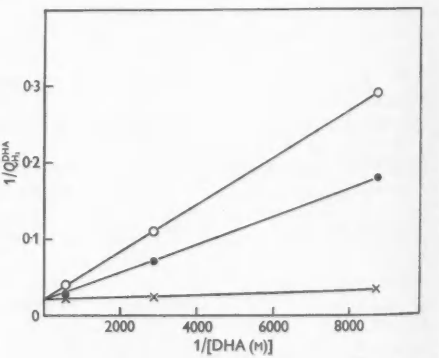


Fig. 6. Reciprocal plot of the effect of various concentrations of L-gulono-γ-lactone and dehydroascorbic acid on the rate of reduction of the dehydroascorbic acid by washed suspensions of *Esch. coli* + 0·02 M glucose at 35°; in 0·15 M phosphate buffer pH 6·2. $Q_{H_2}^{DHA} = \mu$l. H_2 transferred/mg. dry wt./hr. ×—×, no lactone; ●—●, $3{\cdot}5 \times 10^{-3}$ M lactone; ○—○, $7{\cdot}0 \times 10^{-3}$ M lactone.

the rate of reduction of DHA, it is clear that the nature of the groups on carbon atoms 2 and 3 is an important factor. Further, the carboxylic carbonyl group cannot be replaced by a hydrogen atom and a methoxy group, since methyl D-ribofuranoside did not inhibit.

Effect of hydrogen-acceptor concentration on the rate of reduction. The effect of DHA concentration on the rate of reduction was examined using both glucose-grown and lactate-grown cells of the reducing strain of *Esch. coli*. Similar experiments were made with dehydrohydroxytetronic acid using glucose-grown cells. In the experiments with DHA both lactate and glucose were used as hydrogen donors but only glucose for dehydrohydroxytetronic acid.

Fig. 7 shows the curves obtained in a typical experiment, from which the rates were calculated as $Q_{H_2}^{DHA}$. At higher concentrations of DHA the curves are linear; but at lower concentrations the rate of reaction falls with time, and the correspondingly small titrations make it difficult to determine the initial rates accurately.

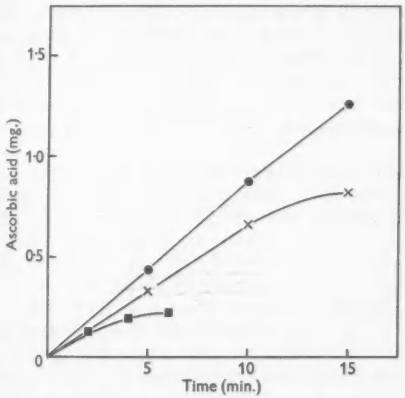


Fig. 7. Effect of dehydroascorbic acid concentration on the rate of its reduction by washed suspensions of glucose-grown *Esch. coli* + lactate (0·02 M) at 35° and pH 6·2 (0·15 M phosphate buffer). ●—●, 1×10^{-3} M DHA; ×—×, $2{\cdot}5 \times 10^{-4}$ M DHA; ■—■, 6×10^{-5} M DHA.

Fig. 8 shows the relations derived from these experiments, fitted Michaelis curves being drawn through the observed points, and Table 2 gives the observed and calculated maximum velocities and the calculated Michaelis constants.

The K_m values for DHA fall clearly into two groups according to the nature of the carbon source on which the cells were grown; the K_m for glucose-grown cells being only about one-tenth of that with cells grown upon lactate. The higher maximum velocities obtained with glucose-grown cells confirmed earlier observations (Eddy, 1952) that cells grown on glucose have much greater DHA-reducing activity than those grown on other sources of carbon.

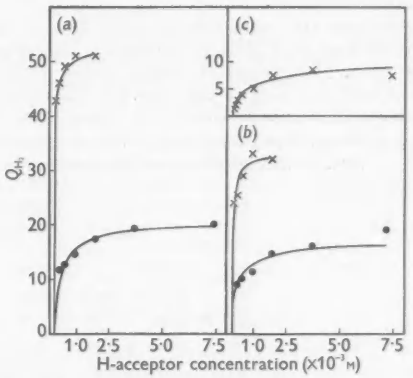


Fig. 8. Relationship between H-acceptor concentration and rate of reduction by washed suspensions of *Esch. coli*: (*a*) dehydroascorbic acid, H-donor glucose; (*b*) dehydroascorbic acid, H-donor lactate; (*c*) dehydrohydroxytetronic acid, H-donor glucose. Temp. 35°, pH 6·2. $Q_{H_2} = \mu$l. H_2 transferred/mg. dry wt./hr. Fitted Michaelis curves are drawn through the experimental points. ×—×, cells grown on glucose; ●—●, cells grown on lactate.

Table 2. *Observed and calculated maximum velocities and the Michaelis constants (K_m values) for the reduction of dehydroascorbic acid and dehydrohydroxytetronic acid by washed suspensions of* Esch. coli

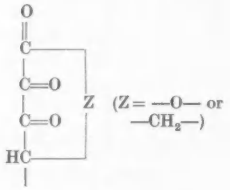
Experiments carried out in 0·15 M phosphate buffer, pH 6·2 under N_2; temp., 35°. $Q_{H_2} = \mu$l. H_2 at s.t.p. transferred/mg. dry wt./hr.

H-acceptor	C-source in growth medium (0·5%, w/v)	H-donor (0·02 M)	Observed maximum velocity Q_{H_2}	Calculated maximum velocity Q_{H_2}	Calculated K_m (M)
DHA	Glucose	Glucose	51	52	3×10^{-5}
DHA	Glucose	Lactate	32	33	5×10^{-5}
DHA	Lactate	Glucose	20	20	3×10^{-4}
DHA	Lactate	Lactate	19	16	3×10^{-4}
Dehydrohydroxy-tetronic acid	Glucose	Glucose	8·5	10	$1{\cdot}5 \times 10^{-3}$

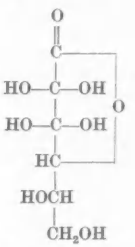
DISCUSSION

Previous workers who have used the dye technique to demonstrate enzymic activation were fortunate in finding that there was no spontaneous reaction between reduced dye and hydrogen acceptor. This has not been the case with DHA which reacts, directly, with leuco-Nile blue. In spite of this difficulty, however, we have been able to show that reducing strains of *Esch. coli* possess a heat-labile enzyme which activates DHA before it is reduced. The potentiometric experiments did not clarify the situation but they confirmed what had been noted with Nile blue; namely that the effect of DHA on the oxidation–reduction potentials of bacterial suspensions was more rapid and greater with the glucose-grown reducing strain than with the lactate-grown reducing strain or with the glucose-grown non-reducing strain.

Although the activation is evidently not absolutely specific to DHA, the specificity is nevertheless close. The groups at carbon atoms 5 and 6 affect only the rate of the reaction. Compounds with a double bond between carbon atoms 2 and 3, such as ascorbic acid, apparently do not interfere. Hence, the specific structure would appear to be



That the γ-lactones of L-gulonic, L-galactonic and D-mannonic acids inhibit the reduction suggests that the diketo grouping is not essential, but that a dihydroxy grouping is effective, and this makes it seem likely that the structure which combines with the enzyme is in fact not that of DHA but of its hydrated form:



This might indeed be expected on general chemical grounds. Though all the possibilities have not been covered, owing to lack of the requisite compounds, it is clear that at least two of the four hydroxyl groups can be replaced by hydrogens, but not all of them; and that the —O— bridge of the ring can be replaced by $—CH_2—$.

Typical Michaelis-Menten relations held for cells grown on different sources of carbon, and supplied with various hydrogen-donating substrates. With lactate-grown cells the maximum rate of reduction of DHA is lower, and the Michaelis constant is higher, than with glucose-grown cells. Hence it seems probable that both the total activity of the enzyme and its affinity for its substrate are affected by the source of carbon used for growth of the bacteria. It is noteworthy that the nature of the substrate subsequently offered to the cells as the primary hydrogen donor is much less important than the carbon source during growth, since the change from glucose to lactate as substrate only produced changes in the Michaelis constant which appeared to be within the experimental error. This might be expected if, as we suppose, the various substrate–dehydrogenase systems reduce cytochrome b_1 which is their common intermediate hydrogen carrier for the DHA-reducing system; while the different maximum rates of DHA reduction, with different substrates, may be a reflexion of the relative ease with which the different dehydrogenase systems are able to reduce cytochrome b_1.

Although the enzyme has not been prepared in a cell-free state, the typical specificity and inhibition relationships, the normal Michaelis-Menten kinetics, and the less critical dye-reduction and potentiometric experiments all indicate that, before it can be reduced by bacteria, DHA must be enzymically activated. For the activating enzyme the name 'dehydroascorbase' seems appropriate, with the reservations about function mentioned below: it emphasizes the analogy with nitratase and tetrathionase, and the alternative dehydroascorbic acid reductase is already in use for a plant enzyme which catalyses the reaction between DHA and reduced glutathione and which is obviously different from the bacterial enzyme. Cytochrome b_1, which is reduced by various dehydrogenases, is an intermediary in the reduction of DHA, but the possibility that there may be additional intermediates is not excluded. In the presence of oxygen as an alternative hydrogen acceptor, the system does not operate unless cyanide is added (Stewart & Sharp, 1945). There is an obvious parallel here to the reduction of nitrate, tetrathionate and trimethylamine oxide, especially as cytochrome b_1 may be implicated in nitrate reduction (Sato & Egami, 1949) but the DHA enzyme is not sensitive to cyanide or azide, nor is it adaptive. The above conclusions apply, strictly speaking, only to *Esch. coli*, but we may assume that other DHA-reducing organisms behave similarly. Further, although the

ability to reduce DHA is restricted to the genus *Staphylococcus* and the family Enterobacteriaceae, apparently because of the limited distribution of cytochrome b_1, there is no other evidence that the systematic distribution of 'dehydroascorbase' is similarly restricted.

On this basis, it seems odd that we should have been able to distinguish so plainly between reducing and non-reducing strains, because there is no apparent reason why strains with intermediate activities of enzyme should not exist, but we have in fact not found any, although a few of our 'non-reducing' strains did reduce DHA very slowly. Nor is it clear why bacteria should possess such an enzyme, since it seems unlikely that DHA would be an important metabolite. Of course, the reduction of DHA might conceivably be the fortuitous activity of an enzyme with a totally different function *in vivo*, but experiments with a variety of substances have not produced any evidence that this is so; also, we have failed to correlate the distribution of ability to reduce DHA, between reducing and non-reducing strains, with any other biochemical activity. The natural significance of the property therefore remains obscure.

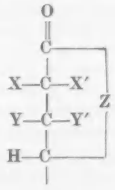
SUMMARY

1. There is a slow chemical reaction between dehydroascorbic acid and reduced Nile blue. This reaction is catalysed by washed cells of some strains of *Esch. coli*.

2. The effect of dehydroascorbic acid on the oxidation–reduction potential of bacterial suspensions was more rapid and greater with a glucose-grown reducing strain than with a lactate-grown reducing strain or a glucose-grown non-reducing strain. With heated cells the changes were greatly retarded.

3. Dehydro-D-araboascorbic, dehydrohydroxytetronic and dehydroreductic acids and 3-hydroxy-1:2-dioxo-3-phenylbutyro-γ-lactone are reduced by a strain of *Esch. coli* which can reduce dehydroascorbic acid.

4. The reduction of dehydroascorbic acid by *Esch. coli* is inhibited competitively by the γ-lactones of L-gulonic, L-galactonic and D-mannonic acids; but not by ascorbic acid, dihydroxymaleic acid, dioxogulonic acid, butyrolactone, D-galacturonic acid methyl ester or methyl D-ribofuranoside. The specific grouping is apparently



where X or X′ and Y or Y′ must be hydroxyl groups and Z can be either —O— or —CH_2—.

5. Michaelis-Menten relationships hold for both dehydroascorbic acid and dehydrohydroxytetronic acid. When the cells are grown on glucose, the maximum velocity is greater and the K_m is lower, than with lactate.

6. These observations suggest that there is a specific thermolabile enzyme which activates DHA (and related substances) before reduction and for which the name 'dehydroascorbase' is proposed.

The authors wish to thank Dr F. A. Isherwood for supplying the lactones and Professor T. Reichstein and Professor H. Dahn for the reductic and hydroxytetronic acids and for the 3-hydroxy-1:2-dioxo-3-phenyl-butyrolactone. The work described in this paper was carried out as part of the programme of the Food Investigation Organization of the Department of Scientific and Industrial Research.

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The Excretion of Synthetic Oestrogens

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In 1939 Stroud investigated the excretion of hexoestrol (*meso*-3:4-di(*p*-hydroxyphenyl)-hexane), stilboestrol (*trans*-3:4-di(*p*-hydroxyphenyl)-hex-3-ene), and dienoestrol (3:4-di(*p*-hydroxyphenyl)-hexa-2:4-diene) when large amounts were administered to rabbits, and later Mazur & Schorr (1942) and Simpson & Wilder Smith (1948), isolated and characterized the corresponding glucuronides.

This was followed by quantitative studies on their urinary excretion at high dose levels (Dodgson, Garton, Stubbs & Williams, 1948; Simpson & Wilder Smith, 1949), while more recently Twombly & Schoenewaldt (1951) and Hanahan, Daskalakis, Edwards & Dauben (1953) using [^{14}C]stilboestrol have demonstrated the importance of the bile and faeces in the excretion of this oestrogen.

It was therefore decided to determine the biological activity of the faeces after administering hexoestrol and stilboestrol to rabbits, and also to carry out a systematic investigation of the effect of dose and route of administration on the ratio of free to conjugated oestrogen eliminated in the urine.

The fate in the rabbit of 17α-ethinyloestradiol, triphenylchloroethylene (1-chloro-1:2:2-triphenylethylene), dipropyldibenzanthracenediol (9:10-dihydroxy-9:10-di-*n*-propyl-9:10-dihydro-1:2:5:6-dibenzanthracene) and 4-*tert*.-amyl phenol has also been investigated; except for the last compound (which was only weakly oestrogenic; Dodds & Lawson, 1938) the amounts present in the urine and faeces have been determined by biological assay. Whenever possible the active compounds were isolated or examined by paper chromatography.

Among these oestrogens only the metabolism of ethinyloestradiol has been previously studied (Stimmel & May, 1951) and these workers could find no detectable amounts of oestrone, oestradiol or oestriol, possible *in vivo* transformation products, in the urine of men following its ingestion. There was evidence, however, of a small amount (not exceeding 3%) of the administered compound in the urine.

EXPERIMENTAL AND RESULTS

General methods

The oestrogens were dissolved or suspended in aqueous ethanol (20%, v/v) for oral administration, and in arachis oil for subcutaneous injection.

A single rabbit was used for each dose with the exception of triphenylchloroethylene where two animals were used.

Single doses were administered up to quantities of 100 mg.; above this, the doses were divided. Thus 1 g. of hexoestrol and stilboestrol were given as 4×250 mg. on consecutive days, 750 mg. of dipropyldibenzanthracenediol also (as four equal doses) over 4 days and 3 g. triphenylchloroethylene as 2×750 mg. to each of two rabbits on consecutive days. 4-*tert*.-Amyl phenol (5 g.) was administered in six equal doses over 12 days.

The animals weighed between 2·5 and 3·5 kg. All were males except for one female (see Table 2). The urine was collected for 3 days and the faeces for 7 days after the last injection; toluene was added to both as a preservative. The urine was stored at 0–5° and the faeces were dried at room temperature and finally *in vacuo* over $CaCl_2$.

Extraction and separation. The urine was made just acid to congo red and continuously extracted with ether for 24 hr. It was assayed as total extract or divided into acidic, phenolic and neutral fractions by repeated hand extraction, first with saturated $NaHCO_3$ solution and then with 2N-NaOH. Both the $NaHCO_3$ and NaOH solutions were acidified to pH 2–3, extracted with ether, the solvent was removed at room temperature *in vacuo* and the residue dissolved in a known volume of absolute ethanol for biological estimation. In some instances the neutralized aqueous solutions were assayed directly.

The dried faeces were crushed, extracted with ether in a Soxhlet extractor for 24 hr. and assayed in 3% aqueous ethanol.

Bioassay. The pure oestrogens in 3% aqueous ethanol (water containing 3%, v/v, ethanol) or arachis oil were administered to groups of five ovariectomized rats, the procedure of subcutaneous injection and vaginal smearing being the standard one used at this Institute (Wilder Smith & Williams, 1948).

This was in order to determine the amount of each substance required to give 50% of positive responses (ED_{50}) (Table 1), which was calculated from the bioassay results by the 'moving averages' statistical method of Thompson (1947).

Rats (45–145) were used for each determination, and it can be seen from Table 1 that the sensitivity of the method depended on the solution medium, lower results being obtained when 3% ethanol instead of arachis oil was used.

Knowing the ED_{50} of each oestrogen, it was now possible to determine their percentage excretion in the rabbit by a process of 'bracketing'. On the assumption that the recovered material contained 10, 25, 50, 75 and 100% of the administered oestrogen, samples calculated to contain the ED_{50} were injected into the rats (five at each dose level) and the approximate range of recovery thus obtained. This range was later narrowed by intermediate dosing. In the results of Tables 2 and 3, quantities corresponding to the lower percentages quoted gave 80–100% response in five

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rats; these percentages are therefore the minimum possible values for recovery. Quantities corresponding to the higher figures gave 0% response in five rats; these percentages therefore represented the maximum possible value for recovery.

In all cases, the extracted urine residue was tested for activity to ensure that extraction was complete, and an extract of 200 ml. of normal male and female rabbit urine was also shown to have no activity.

Examination of compounds and metabolites. All the administered phenolic oestrogens and metabolites with the exception of 4-*tert.*-amyl phenol were examined by paper chromatography (Jellinck, 1953), the system *C* of Bush (1952) being used for oestriol.

Hexoestrol

The glucuronide was separated from the free oestrogen by extracting the ethereal solution, once with 0·5 vol. and twice with 0·25 vol. of saturated $NaHCO_3$ and then twice with 0·25 vol. of water. The pooled aqueous fraction was backwashed twice with 0·25 vol. of ether, which in turn was backwashed twice with 0·25 vol. of bicarbonate solution and once with 0·25 vol. of water. The pooled aqueous fraction was extracted yet once more with twice 0·2 vol. of ether, before acidifying and ether extracting.

As hexoestrol is about 35 times more active than its glucuronide (Table 1), it was essential that the separation should be complete, and two control experiments with a mixture of 50 μg. hexoestrol and 300 μg. hexoestrol glucuronide in 3·5 ml. of absolute ethanol added to a 100 ml. of rabbit urine were carried out to ensure that both the method of extraction and separation were quantitative. The recoveries were 80–110% for hexoestrol and 110–150% for the conjugated oestrogen. The glucuronide of hexoestrol was assayed directly because it was found that refluxing 2 mg. of this conjugate for 1·5 hr. with 15% (v/v) HCl resulted in 60–70% destruction when assayed as free hexoestrol. The addition of zinc dust as used by Smith & Smith (1941) for the natural oestrogens, did not protect it.

Any disulphate of hexoestrol would remain behind in the original aqueous layer at the pH that the continuous extraction had been carried out, as found by Simpson & Wilder Smith (1949), and their method of hydrolysis was adopted. The hydrolysed solution was extracted with ether and assayed in 3% aqueous ethanol. The glucuronide of hexoestrol was isolated by the method of Simpson & Wilder Smith (1948).

Results of recovery experiments are shown in Table 2.

Table 1. *Median effective dose of synthetic oestrogens in ovariectomized rats*

In 3% aqueous ethanol except for values marked *A* which are in arachis oil.

Compound	ED_{50} (μg.)	No. of rats used
Hexoestrol	0·30	145
	0·51 (*A*)	50
Hexoestrol glucuronide	11	65
Stilboestrol	0·27	85
Ethinyloestradiol	0·10	45
	0·25 (*A*)	85
Triphenylchloroethylene	16	90
Dipropyldibenzanthracenediol	11	55

Stilboestrol

This oestrogen was separated from its glucuronide by the process used for hexoestrol, and the percentage excreted in the free form in the urine and faeces determined (Table 3).

Stilboestrol (4 mg.) was obtained by crystallization from benzene from the phenolic and neutral fraction of the urine of the rabbit dosed with 1 g. of the oestrogen. The isolated compound was examined by paper chromatography and was shown to have the same infrared spectrum at 600–2000 cm.$^{-1}$ as pure stilboestrol.

Stilboestrol glucuronide was isolated by the method of Simpson & Wilder Smith (1948).

Ethinyloestradiol

It was shown by paper chromatography that the unchanged oestrogen was present in the faeces, but no oestrone, α-oestradiol or oestriol could be detected there. The urine showed less than 1% activity even when assayed as oestriol, which is the least active member of the natural oestrogen series (ED_{50}, 2·7 μg.), and hydrolysis by refluxing with 15% (v/v) HCl did not increase the activity.

The bioassay results are shown in Table 3.

Table 2. *Hexoestrol recovery values from urine and faeces in the rabbit*

All values as percentage of oestrogen administered; calculated as described on p. 262.

Route of administration	Dose (mg.)	Excreted in the urine: As ethereal sulphate (%)	Excreted in the urine: As mono-glucuronide (%)	Excreted in the urine: As free oestrogen (%)	Excreted in the faeces as free oestrogen (%)	Total recovered (%)
Oral	1	—	18–24	15–25	10–15	43–64
	1	<2	35–45	2–5	4–10	41–60
	1*	<2	40–60	1–2	5–10	46–72
	10	—	6–9	8–13	8–15	22–37
	100	—	4–10	8–15	5–10	17–35
	1000	—	25–40	2–5	<5	27–45
Subcutaneous	1	—	<20	5–12	<10	—
	10	—	25–40	10–15	7–10	42–65
	100	—	5–8	8–12	25–35	38–55
	1000	—	18–25	3–7	4–7	25–39

* Female rabbit.

Table 3. *Recovery of activity of synthetic oestrogens from urine and faeces in the rabbit*

All values as percentage of oestrogen administered; calculated as described on p. 262.

Compound	Route of administration	Dose (mg.)	Activity in urine: Total extract (%)	Activity in urine: Acid fraction (%)	Activity in urine: Phenolic and neutral fraction (%)	Activity in faeces (%)	Total activity recovered (%)
Stilboestrol	Subcutaneous	1	—	—	8–13	35–50	—
		10	—	—	12–20	10–15	—
		100	—	—	12–14	2–5	—
		1000	—	27*	<1	<1	27
Ethinyl-oestradiol	Subcutaneous	40	<1	—	—	12–17	12–17
Triphenyl-chloroethylene	Oral	3000	—	0·75–1·5	<1	16–24	17–25
Dipropyldibenz-anthracenediol	Subcutaneous	750	<1	—	—	3–5	3–5

* Isolated in crystalline form.

Triphenylchloroethylene

Most of the oestrogenic activity was found in the faeces and a small amount in the acid fraction of the urine (Table 3).

All acids and phenols were removed from the faeces extract by repeated extraction with 2N-NaOH, and the neutral ethereal residue was well washed with water and dried over anhydrous Na_2SO_4 before evaporating to dryness. The residue was dissolved in warm absolute ethanol and the impure crystals (198 mg.) which appeared on cooling were filtered and recrystallized from absolute ethanol. A pure crystalline compound (93 mg.) was thus obtained, which did not depress the m.p. of triphenylchloroethylene and which had the same characteristic infrared spectrum in the 'finger print' region (600–2000 cm.$^{-1}$).

The NaOH-soluble fraction of the faeces extract showed less than 5% activity, and the acid fraction of the urine did not give a positive Tollens naphthoresorcinol reaction for glucuronide.

Dipropyldibenzanthracenediol

A small amount of activity was present in the faeces but there was less than 1% activity in the urine (Table 3) which was not increased by hydrolysis for 10 min. with 15% (v/v) HCl. The urine extract did not give a positive Tollens naphthoresorcinol reaction for glucuronide, and all attempts to isolate crystalline material from it were without success.

4-tert.-*Amyl phenol*

This oestrogen was not assayed because it was only weakly active (80 mg./rat) but its *glucuronide* was isolated from the acid fraction of an ether extract by the following method. The $NaHCO_3$ fraction was acidified, extracted with ether and the solvent removed. The tar, which gave a strong positive Tollens naphthoresorcinol reaction for glucuronide, was dissolved in 50 ml. absolute ethanol, boiled with animal charcoal, filtered, and ethanolic KOH (3 g. KOH/100 ml. absolute ethanol) added until no more solid was precipitated. This solid was filtered, dissolved in 50 ml. water, acidified to pH 2–3 and extracted with ether until the aqueous residue gave no colour in the Tollens test. The ether was removed and the syrupy residue crystallized from benzene–acetone (12:1, v/v). Yield: 1·44 g. (13·9% recovery). White crystals m.p. (decomp.) 167–168° after sintering at 70–75° and then gradually darkening in colour.

For the *monohydrate*, found: C, 57·0; H, 7·6. $C_{17}H_{24}O_7$, H_2O requires: C, 57·0; H, 7·3%. Equiv. wt., found: 355; required, 358. Loss in wt. on drying at 110° *in vacuo*: found, 5·2; required, 5·03%.

For the *anhydrous glucuronide*, $C_{17}H_{24}O_7$, found: C, 59·5; H, 6·8. $C_{17}H_{24}O_7$ requires C, 60·0; H, 7·1%.

DISCUSSION

Hexoestrol

The results (Table 2) show that there are very large variations in the amounts excreted as the free and conjugated compounds in the urine and as 'total' oestrogen in the faeces. They also show that neither the dose nor the route of administration seems to have a significant effect on the excretion pattern.

It has been assumed that the amount of activity present in the acid fraction of the extract is solely due to the glucuronide, since on no occasion has more than a trace of ethereal sulphate been found (Dodgson *et al.* 1948; Starnes & Teague, 1949; Simpson & Wilder Smith, 1949), and in any case the latter would not be extracted from the urine by the procedure used (Simpson & Wilder Smith, 1949; Teague & Brown, 1951).

The rabbit is not generally considered suitable for recovery experiments from faeces but hexoestrol appears to be resistant to bacterial attack, as unchanged hexoestrol could be detected in the faeces and high recovery percentages were obtained even with very small amounts of administered oestrogen. This seemed to be also true for the other compounds investigated.

Ethinyloestradiol

The results are similar to those found for men by Stimmel & May (1951) and in addition it has been shown that the faeces are a major route of excretion for this oestrogen in the rabbit.

Triphenylchloroethylene

This compound had been fed in the hope that it would be hydroxylated and excreted in the urine in either the free or conjugated forms or both, as this might have settled the controversy about whether the compound was an oestrogen or pro-oestrogen. All the urinary activity was found in the acid fraction and as no glucuronide could be detected, either a mercapturic acid conjugate of the unchanged oestrogen or an ethereal sulphate, if hydroxylation had taken place, could account for it. Work is in progress at the present to isolate and characterize this compound. The high activity in the faeces is in agreement with the results of Paterson, Gilbert & Gallagher (1949), who on injecting mice intravenously with the radioactive bromo analogue (triphenylbromoethylene) found that most of the organic bromide was in the faeces.

4-tert.-*Amyl phenol*

The fate of 4-*tert.*-amyl phenol in the rabbit was investigated because it is the simplest known compound which has oestrogenic activity, but the only metabolite isolated has been its glucuronide, which as far as is known has not been characterized before.

In conclusion it should be added that in most cases the doses given were far in excess of those needed to show physiological activity but it had been hoped that some metabolites would be isolated and the experiment could then have been repeated with considerably less oestrogen and those metabolites detected and estimated by a micro method.

SUMMARY

1. The excretion pattern of hexoestrol in the rabbit has been determined but it has not been possible to show that either the dose or the route of administration had any marked effect on the ratio of free to conjugated oestrogen in the urine. The percentage excreted as disulphate in the urine is low, even when small doses are administered.

2. Ethinyloestradiol was excreted unchanged mainly in the faeces and no trace of oestrone, α-oestradiol or oestriol could be detected in either the urine or faeces.

3. The percentage of administered stilboestrol, triphenylchloroethylene and dipropyldibenzanthracenediol excreted in the urine and faeces has also been determined. Stilboestrol and its glucuronide were isolated from urine and triphenylchloroethylene recovered unchanged from the faeces.

4. The glucuronide of 4-*tert.*-amyl phenol has been isolated from urine and characterized.

5. It has been shown for hexoestrol and ethinyloestradiol that their ED_{50} as oestrogens varies according to whether they are assayed biologically in arachis oil or 3 % aqueous ethanol, the sensitivity being greater when the latter solvent is used.

I wish to thank Professor Sir Charles Dodds, F.R.S., for having made this work possible, Mr W. Lawson for his help and interest and Mrs S. A. Simpson for her advice on the biological aspects. I am also indebted to Dr A. E. Kellie for the infrared spectra determinations and to Mr H. Jones, assisted by Mrs D. Culliford and Miss J. Mapplethorpe, for carrying out the bioassays.

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Synthesis of D-δ-Amino-δ-carboxyvalerylglycine (a Degradation Product of Cephalosporin N) and of DL-δ-Amino-δ-carboxyvaleramide

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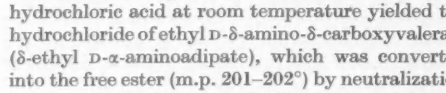
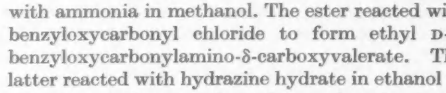
Newton & Abraham (1954) reported that cephalosporin N was probably (D-4-amino-4-carboxy-*n*-butyl)penicillin. On hydrolysis with N-hydrochloric acid at 100° for 6 hr., preparations of cephalosporin N yielded D-α-aminoadipic acid. Less vigorous hydrolysis converted them into a penilloic acid, which gave penicillaminic acid and a neutral aldehyde on treatment with bromine water. The aldehyde was oxidized by silver oxide to an α-aminoadipylmonoglycine. The latter was thought to be δ-amino-δ-carboxyvalerylglycine, because the pK of its amino group was very close to the pK of the α-amino group of α-aminoadipic acid. D-δ-Amino-δ-carboxyvalerylglycine (I) has now been synthesized and appears to be identical with the natural product.

The synthesis was effected without difficulty by a procedure similar to that used by Hegedüs (1948) and by LeQuesne & Young (1950) for the synthesis of γ-glutamyl peptides. Although Sachs & Brand (1954) have reported that this procedure yields a mixture of α- and γ-glutamyl peptides, no α-peptide of α-aminoadipic acid appeared to be present in the recrystallized product.

Treatment of D-α-aminoadipic acid (Greenstein, Birnbaum & Otey, 1953) with ethanol containing hydrochloric acid at room temperature yielded the hydrochloride of ethyl D-δ-amino-δ-carboxyvalerate (δ-ethyl D-α-aminoadipate), which was converted into the free ester (m.p. 201–202°) by neutralization with ammonia in methanol. The ester reacted with benzyloxycarbonyl chloride to form ethyl D-δ-benzyloxycarbonylamino-δ-carboxyvalerate. The latter reacted with hydrazine hydrate in ethanol to yield crystalline D-δ-benzyloxycarbonylamino-δ-carboxyvalerylhydrazide (II) (m.p. 132–133°).



(I)



(II)

The azide formed from (II) was coupled with glycine ethyl ester in chloroform solution. The resulting D-δ-benzyloxycarbonylamino-δ-carboxyvalerylglycine ester was hydrolysed by N sodium hydroxide to the corresponding acid, and the latter was converted into D-δ-amino-δ-carboxyvalerylglycine (m.p. 218–219°) by hydrogenation in the presence of palladium black.

D-δ-Amino-δ-carboxyvalerylglycine yielded one molecular proportion of carbon dioxide in the ninhydrin–carbon dioxide method of Van Slyke, Dillon, MacFadyen & Hamilton (1941) for the determination of α-amino acids. Electrometric titration at 20° showed that it contained a basic group with a pK of 9·8 and two acidic groups with pK values of $<3{\cdot}0$ and 3·8 respectively. Similar values had been found for the natural product (Newton & Abraham, 1954). On paper chromatograms run with butanol–acetic acid (Woiwod, 1949) the synthetic and natural products showed the same R_F value, which was close to that of glycine. When subjected to ionophoresis on paper at pH 7 (Newton & Abraham, 1954) they migrated towards the anode at the same rate. Dr Dorothy Hodgkin and Mrs M. Mackay reported that 'X-ray powder photographs of the two products were identical, apart from one or two small details of line structure which might be due to traces of impurity in the natural preparation'.

An attempt to prepare DL-δ-amino-δ-carboxyvaleramide by the action of aqueous ammonia on ethyl DL-δ-amino-δ-carboxyvalerate led to the formation of material that gave no colour with ninhydrin and consisted mainly of 6-oxopiperidine-2-carboxylic acid. The desired compound was obtained by conversion of ethyl DL-δ-benzyloxycarbonylamino-δ-carboxyvalerate into the corresponding amide and removal of the benzyloxycarbonyl group. Electrometric titration showed that it contained an acidic group with a pK of 2·6 and a basic group with a pK of 9·7. These values indicated that the δ-carboxyl group of α-aminoadipic acid was involved in amide formation.

D-δ-Amino-δ-carboxyvalerylglycine, like γ-glutamylpeptides, is readily hydrolysed by acid. It appeared to be largely split into α-aminoadipic acid and glycine by treatment with N hydrochloric acid at 105° for 6 hr. When heated in water alone it

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yielded glycine but very little α-aminoadipic acid. Presumably autohydrolysis had occurred in a manner similar to that observed by LeQuesne & Young (1952) with γ-glutamylpeptides, leading in the present case to the formation of glycine and 6-oxopiperidine-2-carboxylic acid. DL-δ-Amino-δ-carboxyvaleramide also appeared to yield 6-oxopiperidine-2-carboxylic acid on heating in aqueous solution. Under similar conditions glutamine has been reported to yield mainly pyrrolidonecarboxylic acid (Chibnall & Westall, 1932; Melville, 1935).

EXPERIMENTAL

Ethyl D-δ-*amino*-δ-*carboxyvalerate.* D-α-Aminoadipic acid (360 mg.) (kindly supplied by Dr J. P. Greenstein) was dissolved in 1·33 N ethanolic HCl (5 ml.). After being kept at 20° for 30 min., the solution was evaporated to dryness *in vacuo*. The residual ester hydrochloride was washed with ether, dissolved in methanol (2·5 ml.) and the soluton neutralized with 35% (w/v) NH_3. The ester formed a crystalline precipitate (316 mg.) which was filtered and washed with methanol (1 ml.). It had m.p. 201–202°, unchanged by recrystallization from aqueous ethanol. (Found: C, 50·7; H, 8·0. $C_8H_{15}O_4N$ requires C, 50·8; H, 7·9%.)

Ethyl D-δ-amino-δ-carboxyvalerate behaved as a neutral substance when subjected to ionophoresis on paper at pH 7 (Newton & Abraham, 1954). On paper chromatograms run with butanol–acetic acid (Woiwod, 1949) it showed an R_F value of 0·58.

Ethyl DL-δ-*amino*-δ-*carboxyvalerate.* DL-α-Aminoadipic acid (348 mg.) (kindly supplied by Dr R. Gaudry) was esterified under the conditions described in the preceding experiment. The product (290 mg.) had m.p. 190°, raised by recrystallization from aqueous ethanol to 194–195°. (Found: C, 50·8; H, 7·7. $C_8H_{15}O_4N$ requires C, 50·8; H, 7·9%.)

Ethyl D-δ-*benzyloxycarbonylamino*-δ-*carboxyvalerate.* Ethyl D-δ-amino-δ-carboxyvalerate (290 mg.) was dissolved in water (2 ml.) containing $NaHCO_3$ (150 mg.). The solution was stirred at 0° while benzyloxycarbonyl chloride (280 mg.) and N-NaOH (1·6 ml.) were added slowly during 20 min. The mixture was stirred for a further 30 min. at room temperature and then extracted with 1 vol. of ether. The aqueous solution was brought to pH 2 with 5 N-HCl and extracted twice with 1 vol. of ether. The ether extract was dried over Na_2SO_4. On evaporating the ether, the benzyloxycarbonyl derivative remained as a syrup. It was precipitated from ether solution by light petroleum as an oil (350 mg.).

Ethyl DL-δ-*benzyloxycarbonylamino*-δ-*carboxyvalerate.* Ethyl DL-δ-amino-δ-carboxyvalerate (190 mg.) was allowed to react with benzyloxycarbonyl chloride under the conditions described in the preceding experiment. The product (271 mg.) was obtained as a crystalline mass. It had m.p. 89–90°, unchanged by recrystallization from a mixture of ether and light petroleum. (Found: C, 59·5; H, 6·4. $C_{16}H_{21}O_6N$ requires C, 59·5; H, 6·5%.)

D-δ-*Benzyloxycarbonylamino*-δ-*carboxyvalerylhydrazide.* Ethyl D-δ-benzyloxycarbonylamino-δ-carboxyvalerate (350 mg.) was dissolved in ethanol (0·9 ml.) and the solution added drop by drop to 0·38 ml. of 100% (w/w) hydrazine hydrate. The mixture was left at room temperature for 48 hr. and most of the ethanol was removed in a stream of air at 30°. The remaining solution was adjusted to pH 2 with 2 N-HCl and a saturated aqueous solution of sodium acetate was then added until the pH rose to 4·7. The product began to crystallize. After the mixture had been kept for 4 hr. at 0°, the hydrazide was filtered, washed with water, and dried (190 mg.). It had m.p. 128–130°, raised by recrystallization from hot water to 132–133°. (Found: C, 54·0; H, 6·2; N, 13·7. $C_{14}H_{19}O_5N_3$ requires C, 54·3; H, 6·2; N, 13·6%.)

D-δ-*Benzyloxycarbonylamino*-δ-*carboxyvalerylglycine.* The hydrazide from the preceding experiment (168 mg.) was dissolved in water (4 ml.) containing 11 N-HCl (0·193 ml.). $CHCl_3$ (3 ml.) was added and the mixture stirred at 0°. A solution of $NaNO_2$ (60 mg.) in water (0·5 ml.) was added dropwise during 5 min. and stirring continued for a further 10 min. The $CHCl_3$ solution of the azide was separated, washed twice with ice-cold water and dried for a few seconds over Na_2SO_4. It was then added to a solution of glycine ethyl ester (prepared from 660 mg. of glycine ethyl ester hydrochloride by the method of Hillmann, 1946) in $CHCl_3$ at 0°. The mixture was kept for 30 min. at 0° and then for 18 hr. at room temperature. It was then washed successively with 2 N-HCl and with water and dried over Na_2SO_4. Evaporation of the $CHCl_3$ *in vacuo* left the ethyl ester of D-δ-benzyloxycarbonylamino-δ-carboxyvalerylglycine as a syrup (200 mg.). It was converted into the corresponding acid by dissolving in N-NaOH (1·7 ml.) and keeping the solution at room temperature for 2 hr. The solution was acidified with 5 N-HCl and extracted 4 times with ethyl acetate (3·5 ml.). The ethyl acetate was dried over Na_2SO_4 and evaporated *in vacuo*. The product remained as an oil (127 mg.). It was precipitated as an oil from ethyl acetate by light petroleum.

D-δ-*Amino*-δ-*carboxyvalerylglycine.* The product from the previous experiment (126 mg.) was dissolved in 50% (v/v) methanol (2·5 ml.) containing glacial acetic acid (0·06 ml.). Palladium black (30 mg.) was added and H_2 passed through the mixture. The evolution of CO_2 ceased in 2 hr. The solution was separated from the catalyst and evaporated *in vacuo*, leaving a crystalline residue (76 mg.). After recrystallization from aqueous ethanol, D-δ-amino-δ-carboxyvalerylglycine (60 mg.) had m.p. 218–219°; $[\alpha]_D^{20}$ – 6·0° in water (*c*, 3·4). (Found: C, 44·0; H, 6·3; N, 13·1. $C_8H_{14}O_5N_2$ requires C, 44·0; H, 6·4; N, 12·9%.) On mixing with the corresponding compound (m.p. 213–214°) from cephalosporin N, the m.p. was 214–215°.

DL-δ-*Benzyloxycarbonylamino*-δ-*carboxyvaleramide.* Ethyl DL-δ-benzyloxycarbonylamino-δ-carboxyvalerate (200 mg.) was dissolved in 35% (w/w) NH_3 (2·5 ml.) and the solution kept at 18° for 48 hr. The solution was then evaporated to dryness *in vacuo*, the residue dissolved in 0·5 ml. of water, and glacial acetic acid gradually added. A crystalline precipitate began to form when the pH of the solution had fallen to 3·7. After 1 hr. the crystals were filtered, yielding 88 mg. of product which had m.p. 168–170°. A second crop (62 mg.), which had m.p. 160–170°, was obtained by acidifying the filtrate to pH 3·0. The two crops were combined and recrystallized from 50% (w/w) ethanol, yielding 135 mg. of fine needles which had m.p. 172–173°. (Found: C, 57·0; H, 6·1; N, 9·5. $C_{14}H_{18}O_5N_2$ requires C, 57·1; H, 6·1; N, 9·5%.)

DL-δ-*Amino*-δ-*carboxyvaleramide.* DL-δ-Benzyloxycarbonylamino-δ-carboxyvaleramide (113 mg.) was dissolved in 50% (v/v) methanol (4·5 ml.) containing glacial acetic acid (0·06 ml.) and hydrogenated in the presence of

palladium black (30 mg.). The product (56 mg.) was recrystallized from aqueous ethanol. It had m.p. 195–196°. (Found: C, 44·6; H, 7·4; N, 17·9. $C_6H_{12}O_3N_2$ requires C, 45·0; H, 7·5; N, 17·5%.)

This product behaved as a neutral compound when subjected to ionophoresis on paper at pH 7 (Newton & Abraham, 1954). It appeared, however, to contain a trace of α-aminoadipic acid. On paper chromatograms run with butanol–acetic acid (Woiwod, 1949) it occupied the same position as aspartic acid ($R_F = 0{\cdot}18$) and on chromatograms run with phenol–HCN it showed an R_F value of 0·67. In both cases the R_F values were slightly greater than those for glutamine.

Autohydrolysis. D-δ-Amino-δ-carboxyvalerylglycine (1 mg.) was dissolved in water (1 ml.) and the solution heated in a sealed tube at 105° for 24 hr. Samples of the product (20 μg.) were subjected to ionophoresis on paper in a collidine acetate buffer, pH 7·0 (Newton & Abraham, 1954), and to chromatography on paper in butanol–acetic acid (Woiwod, 1949). When developed with ninhydrin, both papers showed a strong spot in the position occupied by glycine, but only a very weak spot in the position occupied by α-aminoadipic acid. No spot corresponding to the original peptide was visible. When the peptide was hydrolysed in 6N-HCl at 105° for 20 hr. the product showed two strong spots corresponding to glycine and α-aminoadipic acid in approximately equimolecular proportions. The spots were only slightly less intense when the same amount of peptide was hydrolysed in N-HCl at 105° for 6 hr.

DL-δ-Amino-δ-carboxyvaleramide (1 mg.) was dissolved in water (0·25 ml.), and the solution heated at 105° in a sealed tube for 18 hr. A sample (20 μg.) was subjected to ionophoresis on paper at pH 7 (Newton & Abraham, 1954). Development with ninhydrin showed no spot corresponding to the neutral amide and only a trace of material in the position occupied by α-aminoadipic acid. When the amide was heated in N-HCl at 105° for 6 hr. the product showed a single strong spot in the position occupied by α-aminoadipic acid.

SUMMARY

1. D-δ-Amino-δ-carboxyvalerylglycine has been synthesized by a process in which D-α-aminoadipic acid was converted, through its δ-ester, into the azide of D-δ-benzyloxycarbonylamino-δ-carboxyvaleric acid and the latter coupled with glycine ethyl ester. The synthetic substance appears to be identical with a product obtained by degradation of cephalosporin N.

2. DL-δ-Amino-δ-carboxyvaleramide has been synthesized by conversion of the ethyl ester of DL-δ-benzyloxycarbonylamino-δ-carboxyvaleric acid into the corresponding amide and removal of the benzyloxycarbonyl group.

3. D-δ-Amino-δ-carboxyvalerylglycine and DL-δ-amino-δ-carboxyvaleramide are readily hydrolysed by hot dilute hydrochloric acid with the liberation of α-aminoadipic acid. When heated in water alone they are hydrolysed without the formation of α-aminoadipic acid. In the latter case 6-oxopiperidine-2-carboxylic acid is presumably one of the main products of hydrolysis.

Analyses were by Weiler and Strauss. We are grateful to Mrs A. Giles and Mr O. Boys for technical assistance.

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The Formation of Urocanic Acid and Glutamic Acid in the Fermentation of Histidine by *Clostridium tetanomorphum*

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Woods & Clifton (1937) showed that washed-cell suspensions of *Clostridium tetanomorphum* ferment various single amino acids, and that L-glutamic acid and L-histidine each yield hydrogen, carbon dioxide and ammonia, together with steam-volatile fatty acids. In the case of glutamic acid, these last consisted of butyric and acetic acids in the molar ratio of 1:3; the fatty acids arising from histidine were not identified. The production of more than 2 moles NH_3/mole histidine decomposed indicated that disruption of the glyoxaline ring of histidine had occurred at some stage in the fermentation. Some years previously, Edlbacher & Kraus (1930) had obtained evidence that glutamic acid was an intermediate in the catabolism of histidine by mammalian liver, and these observations, taken in

conjunction with their own results, led Woods & Clifton (1937) to suggest that glutamic acid was an intermediate in the fermentation of histidine by *Cl. tetanomorphum*. Furthermore, Edlbacher & Neber (1934) had contended that the first step in histidine decomposition *in vivo* involved rupture of the glyoxaline ring rather than deamination in the α-amino position. The work now described was undertaken to obtain more direct evidence concerning the steps in histidine fermentation by *Cl. tetanomorphum*, and it was found that urocanic acid and glutamic acid are intermediates in this fermentation (Wickremasinghe, 1952). Simultaneously and independently, Hall (1952) and Tabor & Hayaishi (1952) showed that urocanic acid was the first intermediate in the catabolism of histidine by mammalian liver and *Pseudomonas fluorescens* respectively.

MATERIALS AND METHODS

Organisms, growth media and preparation of washed-cell suspensions. The organism used was *Clostridium tetanomorphum* National Collection of Type Cultures (N.C.T.C.), no. 500. *Clostridium welchii* SR 12 (N.C.T.C. no. 6784), *Cl. welchii* BW 21 (N.C.T.C. no. 6785) and *Escherichia coli* K 4 (Biochemistry Department, Sheffield University) were used as sources of glutamic acid decarboxylase (Gale, 1945), histidine decarboxylase (Gale, 1945) and formic hydrogenlyase (Woods, 1936) respectively. Stock cultures of *Cl. tetanomorphum* were maintained in the dried state *in vacuo* (Stamp, 1947) and subcultures were grown in Robertson's cooked meat medium. For bulk cultures, the medium, having initial pH 7 and consisting of 1% (w/v) Pronutrin (an enzymic hydrolysate of casein prepared by Herts Pharmaceuticals Ltd., Welwyn), 0·1% (w/v) Marmite and 20 g. minced ox heart/l., was dispensed in flasks, autoclaved, cooled rapidly, and after the aseptic addition of sterile solutions of $FeSO_4$ in N-H_2SO_4 (final concn. 6 mg. Fe^{2+}/l.) and Na_2S, $9H_2O$ (final concn. 0·01%, w/v), each litre of medium was inoculated with 4 ml. of the broth from a 15 hr. subculture. The flasks were then incubated anaerobically in H_2 containing 5% CO_2 (v/v) for 15 hr. at 37°. *Esch. coli* was grown in a medium (initial pH 7) composed of 1% (w/v) Pronutrin, 5% yeast autolysate (Barker & Beck, 1942) (i.e. the equivalent of 5 g. autolysed yeast/100 ml.) and 2% (w/v) glucose. Each litre of medium was inoculated with 5 ml. of a 15 hr. subculture grown in the same medium, and then incubated anaerobically for 12 hr. at 37°. Cultures were harvested by centrifuging, washed once with 0·9% (w/v) NaCl (freshly boiled and cooled in the case of *Cl. tetanomorphum*) and suspended in the same saline solution to give suspensions containing approx. 30 mg. dry wt. of cells/ml. Suspensions of *Cl. tetanomorphum* must be used immediately, since exposure to air soon leads to a decline in the ability to form H_2 and CO_2 from histidine (cf. Woods & Clifton, 1937).

Preparation of acetone powders of Cl. tetanomorphum. The washed-cell suspension was run into twice its volume of acetone, previously cooled to 4°, and allowed to stand for 30 min. in an ice bath. The insoluble material was collected by filtration and washed successively with acetone, a mixture of acetone and ether (1:1, v/v) and, finally, ether (all previously cooled to 4°). The powder obtained in this way contained the enzymes responsible for the first steps in the decomposition of histidine, and retained its activity for several weeks when stored in a desiccator.

Chemicals. L-Histidine and L-glutamic acid were used throughout (L. Light and Co. Ltd., Colnbrook, Bucks). Urocanic acid was prepared from histidine by the method of Edlbacher & von Bidder (1942).

Sørensen phosphate buffers were prepared from solutions of KH_2PO_4 and Na_2HPO_4 (Sørensen, 1912).

Analytical procedures

Hydrogen and carbon dioxide were determined manometrically using Warburg manometers; when both H_2 and CO_2 were produced, the amounts of each were determined by the 'indirect method' used by Woods & Clifton (1937).

Ammonia. Free NH_3 was estimated by the vacuum-distillation technique of Parnas & Heller (1924), and the sample, in a total volume of 8 ml., was made alkaline by the addition of 2 ml. of a solution containing 5% (w/v) $NaBO_2$ and 10% (w/v) K_2CO_3. These conditions are not sufficiently alkaline to disrupt any alkali-labile bonds which on hydrolysis yield NH_3. This 'alkali-labile' ammonia was estimated by the micro-diffusion technique of Conway & O'Malley (1942) by adding an equal volume of 10N-NaOH to the sample in the outer chamber of the Conway unit. In both procedures the NH_3 was absorbed in boric acid containing a mixed indicator (Conway & O'Malley, 1942), and was estimated by titration with standard HCl from a microburette.

Detection, identification and estimation of steam-volatile fatty acids. The steam-volatile fatty acids were separated from experimental fluids acidified with H_2SO_4 (final concn. about 2N) by steam distillation in the apparatus of Markham (1942). The distillate was aerated with CO_2-free air for 10 min. and the total volatile acid present determined by titration with standard CO_2-free NaOH using phenol red as the indicator. The titrated distillate was evaporated to dryness and the residue of sodium salts of the fatty acids was dissolved in a known volume of water (solution *A*). Samples of solution *A* were analysed qualitatively for the presence of the lower volatile fatty acids using a paper-chromatography technique (Elsden & Lewis, 1953) based on the methods of Brown (1950), Brown & Hall (1950) and Hiscox & Berridge (1950). A sample of solution *A* was taken and formic acid was detected and estimated manometrically in terms of the H_2 released by a washed-cell suspension of *Esch. coli* possessing formic hydrogenlyase activity (cf. Woods, 1936). These determinations were carried out at pH 6·5 in 0·08M Sørensen phosphate buffer under a gas phase of O_2-free N_2 with KOH and filter paper in the centre wells of the cups to absorb CO_2. The formic acid in the remaining part of solution *A* was destroyed by distillation from $MgSO_4$ and $HgSO_4$ in the presence of H_2SO_4 (Friedemann, 1938). As before, after the total amount of volatile acid in the distillate had been determined by titration with standard CO_2-free NaOH, the titrated distillate was evaporated to dryness and the residue dissolved in 0·1 ml. water (solution *B*). The fatty acids present in this concentrated solution were identified and their relative proportions determined by the quantitative gas–liquid partition-chromatography technique of James & Martin (1952). Since the total amount of volatile acids in the distillate from which solution *B* had been prepared was known, the absolute

amounts of each of the fatty acids in the original experimental sample could be readily determined.

Detection of compounds containing amino and glyoxaline groups. Samples of the experimental systems were analysed qualitatively for amino and glyoxaline compounds by descending paper chromatography using Whatman no. 4 paper and *n*-butanol–acetic acid–water (upper layer from 30 ml. glacial acetic acid plus 250 ml. of 50/50 (v/v) mixture of *n*-butanol and water) (Woiwod, 1949), or phenol–water (Consden, Gordon & Martin, 1944) as the mobile phase. Amino compounds in the dried chromatogram were located by spraying with a solution of ninhydrin in $CHCl_3$. Glyoxaline compounds appeared as red spots when a duplicate chromatogram was sprayed with 10% (w/v) Na_2CO_3 followed by diazotized sulphanilic acid (1 vol. 5% (w/v) $NaNO_2$ added to 1 vol. 1% (w/v) sulphanilic acid in N-HCl, and, after 5 min., another 4 vol. 5% (w/v) $NaNO_2$).

Amino nitrogen was estimated by the method of Pope & Stevens (1939).

L-Glutamic acid and L-histidine were each estimated by the appropriate specific bacterial amino acid decarboxylase (Gale, 1945). L-Histidine was also determined in some experiments by the unspecific colorimetric procedure of Macpherson (1946). All glyoxaline compounds form a coloured complex in this procedure, but since the absorption coefficients of the complexes differ, quantitative results can only be obtained if the samples for analysis contain known compounds and the appropriate standards are available.

DL-Glutamic acid was determined by the method of Cohen (1939) after precipitating proteins from the samples with freshly prepared 5% (w/v) metaphosphoric acid.

Microhydrogenation of compounds containing carbon-to-carbon double bonds was followed manometrically (Harrison, 1939), using colloidal Pd as the catalyst under an atmosphere of H_2.

Unless stated to the contrary, all experiments were performed at 37° and quantities are expressed in terms of μmoles.

Melting points are uncorrected.

Spectrophotometric measurements were made with a Unicam Ultraviolet Spectrophotometer (Model SP500) with quartz cells having a light path of 1 cm. Extinction coefficients are given in terms of $E^{1\%}_{1\,cm.}$.

RESULTS

Products of histidine fermentation by washed-cell suspensions. Washed-cell suspensions of *Cl. tetanomorphum* were incubated anaerobically under N_2 with histidine and 0·04M Sørensen phosphate buffer (pH 7·2) in Warburg manometers. When the rate of production of H_2 and CO_2 was equal to that in the controls which contained no histidine, acid was tipped from the second sidebulb in order to estimate bound CO_2 and to stop the reaction. The contents of the two manometers containing histidine (i.e. one without and one with a CO_2-absorber for the measurement of CO_2 and H_2 in the former and H_2 alone in the latter) were transferred quantitatively to a measuring cylinder and made to a known volume: the two 'control' manometers were treated similarly. The results of the analysis of samples for NH_3, total volatile acid and residual histidine (Table 1) were similar to those of Woods & Clifton (1937).

The titrated steam distillate containing the volatile fatty acids was concentrated (solution *A*) and since redistillation of a sample from a mixture of $HgSO_4$, $MgSO_4$ and H_2SO_4 (Friedemann, 1938) appreciably reduced the amount of fatty acid in the distillate, it was suspected that solution *A* contained formic acid. Solution *A* was therefore assayed for this acid by means of formic hydrogenlyase in the form of a washed-cell suspension of *Esch. coli*, and both H_2 and CO_2 were produced. Apart from formic acid, pyruvic acid is the only steam-volatile substance which would yield H_2 and CO_2 in such conditions, and since no pyruvic acid could be detected by the colorimetric procedure of Lichstein & Umbreit (1947), it was concluded that the H_2 and CO_2 arose from formate. The amount of H_2 corresponded to the formation of approximately 1 mole formic acid/mole histidine fermented (Table 1) and accounted for 35% of the total volatile fatty acids in the fermentation products. The formic

Table 1. *Products of histidine fermentation by washed-cell suspensions of* Cl. tetanomorphum

Complete systems contained 0·04M Sørensen phosphate buffer (pH 7·2), 12·5 mg. dry wt. of washed cells in 0·5 ml. 0·9% (w/v) NaCl (tipped from sidebulb after equilibration period) and 25 μmoles histidine; total volume, 2·5 ml. Control systems: no histidine. Hydrogen determined in pair of manometers containing 0·1 ml. 20% (w/v) KOH and filter paper in centre well. Carbon dioxide measured in presence of H_2 using double-sidebulb manometers; initial and final bound CO_2 determined by tipping acid at beginning and end of experimental period (150 min.). Gas phase, O_2-free N_2; temp., 37°. NH_3 estimated by Conway procedure using NaOH (final concn. 5N): residual histidine by decarboxylase method. For other determinations, see text.

	μmoles			μmole product/μmole histidine	
Product	Control system A	Complete system B	Product due to histidine (B – A)	Present work	Woods & Clifton (1937; 1938)
H_2	4·9	6·7	1·8	0·07	0·14
CO_2	1·2	20·9	19·7	0·79	0·67
NH_3	20·3	77·4	57·1	2·30	2·18
Residual histidine	0·0	0·0	0·0	—	—
Volatile fatty acids					
Total	13·6	75·9	62·3	2·49	Present
Formic acid in soln. *A*	3·2	24·9	21·7	0·87	—
Total in soln. *B*	10·1	49·3	39·2	1·55	—
Acetic acid	10·1	39·5	29·4	1·18	—
Butyric acid	0·0	9·8	9·8	0·38	—

acid in a sample of solution *A* was removed and a concentrated solution, *B*, containing the other fatty acids was prepared. Examination by paper chromatography showed that acetic and butyric acids were present, and these were estimated quantitatively by the technique of James & Martin (1952).

Edlbacher & Kraus (1930) had obtained evidence that formic acid was produced in the catabolism of histidine in liver and that certain liver preparations formed an intermediate which on chemical hydrolysis yielded glutamic acid and formic acid. Hence, in the *Cl. tetanomorphum* experiments, the formation of formic acid and of acetic and butyric acids in the proportion of 3:1 from both glutamic acid (Woods & Clifton, 1938) and histidine (Table 1), provided supporting evidence for the suggestion of Woods & Clifton that glutamic acid is an intermediate in the fermentation of histidine.

Detection of intermediates in histidine fermentation by paper chromatography. Washed-cell suspensions of *Cl. tetanomorphum* were incubated anaerobically for 3 hr. with histidine in Sørensen phosphate buffer (pH 7·2) and samples were withdrawn at 10 min. intervals and the reaction stopped by the addition of acid. The cells were removed by centrifuging and the supernatants were neutralized and then analysed by paper chromatography for the presence of amino and glyoxaline compounds. When butanol–acetic acid–water was used for development, it was found that the first samples contained two glyoxaline compounds with R_F values approx. 0·11 and 0·56 at 20°. The former corresponded to that for histidine, and in a duplicate chromatogram only this compound gave a colour with ninhydrin, indicating that the other glyoxaline compound (compound *X*) no longer contained an α-amino group. If the same amounts of the supernatants were placed on the chromatograms, it was seen that in the early stages of the reaction period histidine disappeared and that the amount of the compound *X* (R_F 0·56) increased. In the later stages of the fermentation this compound gradually disappeared from the system. Chromatograms sprayed with a solution of ninhydrin also revealed that some of the samples taken about the middle of the reaction period contained a ninhydrin-positive substance with R_F values in the butanol–acetic acid–water and phenol–water systems corresponding to those of glutamic acid. Evidence was thus obtained that the fermentation of histidine involved a non-amino glyoxaline compound and glutamic acid.

Preliminary experiments with acetone powders. In order to simplify the experimental system with a view to aiding the study of the mode of formation and the identification of the unknown glyoxaline compound, a comparison was made of the products formed by a washed-cell suspension and by an acetone powder (prepared from the same suspension) when each was incubated anaerobically with histidine or glutamic acid for 2·5 hr. at 37° in 0·04 M Sørensen phosphate buffer (pH 7·2). The acetone powder had no action on glutamic acid, and though no H_2, CO_2, acetic or butyric acid was produced, histidine was attacked with the formation of 1 mole NH_3/mole histidine and the system no longer gave the diazo reaction for glyoxaline compounds. Such preliminary experiments showed that an acetone powder of *Cl. tetanomorphum* was suitable for studying the initial steps in histidine fermentation. Since nothing was known about the enzyme systems concerned in the various reactions, it was decided to use the whole acetone powder rather than preparations of enzyme systems extracted from it.

Incubation of the acetone powder with histidine for only a short period (10 min.) led to the formation of the glyoxaline compound *X*, the existence of which was demonstrated by paper chromatography and by the fact that the quantity of glyoxaline compounds in the system, as measured by the method of Macpherson (1946) using histidine as a standard, was greater than could be accounted for in terms of residual histidine as determined by the specific decarboxylase technique (cf. Fig. 1). The effect of pH in the range 6·5–10·4 was studied by incubating anaerobically samples of the acetone powder with the appropriate buffer system of $NaHCO_3$ and CO_2 (cf. Krebs, 1935) or Na_2CO_3 and $NaHCO_3$ (Warburg, 1919). The reaction was stopped after 10 min. by the addition of acid and it was found that the disappearance of histidine and the production of NH_3 were greatest at pH 9·5. Moreover, the close agreement between the values obtained for residual histidine and amino nitrogen, as well as the equivalence between the amounts of NH_3 produced and histidine decomposed, led to the conclusion that the initial step in histidine degradation is concerned with the α-amino group and not with the glyoxaline part of the molecule and that in this reaction 1 mole of histidine gave rise to 1 mole of the compound *X* and 1 mole of NH_3.

During some preliminary experiments, borate instead of carbonate buffers were used in the pH range 8–10. Paper chromatography revealed the presence of three glyoxaline compounds; two of these were compound *X* and histidine. The third compound remained on or near to the starting

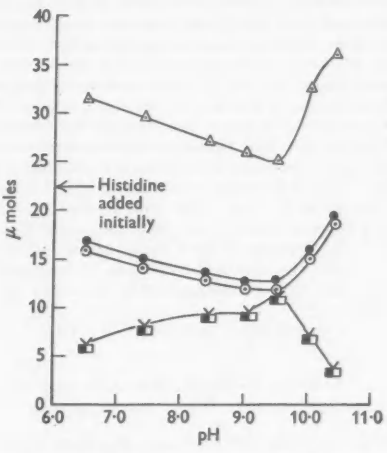

Fig. 1. Effect of pH on degradation of histidine by an acetone powder of *Cl. tetanomorphum*. Complete systems contained 0·04 M-$NaHCO_3$ plus Na_2CO_3 as required, 5 mg. acetone powder and 22·3 μmoles histidine; total volume 2·5 ml. Control systems: no histidine. Gas phase O_2-free N_2 or N_2–5% (v/v) CO_2 as required. Temp. 37°. NH_3 estimated by Parnas technique (×) and by the Conway procedure (■); □, histidine decomposed (i.e. histidine added − residual histidine); ⊙, residual histidine, by decarboxylase procedure; ●, amino nitrogen, assuming that only amino compound present was histidine; △, glyoxaline by Macpherson's technique and using histidine as standard. Values in control systems subtracted.

line of the chromatograms and was subsequently found to be due to the formation of a complex between borate and urocanic acid. Such a result emphasizes that misleading information may be obtained from experimental systems containing borate buffers.

After prolonged incubation of the acetone powder with histidine, the system no longer gave the diazo reaction for glyoxaline compounds. When a mild alkali was used in the procedure for determining the amount of NH_3 in such systems, the latter were found to contain 1 mole free NH_3/mole histidine decomposed, whereas if a strong alkali was used (e.g. NaOH, final concn. 5N), a further mole of NH_3 was recovered. This result indicated the presence of a substance (compound *Y*) containing two nitrogen atoms, one of which was bound in an alkali-labile linkage and which on hydrolysis yielded NH_3. Compound *Y* was formed from histidine without the production of H_2 or CO_2 and solutions containing it did not exhibit an absorption peak in the ultraviolet region of the spectrum (cf. urocanic acid, below). In keeping with the latter result was the observation that the system did not contain substances with carbon-to-carbon double bonds reducible by microhydrogenation under the conditions described by Harrison (1939). Acid or alkaline hydrolysis at 100° of solutions containing compound *Y* yielded 0·5 mole L-glutamic acid (estimated by decarboxylase method) and 1 mole α-amino nitrogen (Pope & Stevens, 1939)/mole histidine decomposed. Since paper chromatography showed the presence of only one amino compound, i.e. glutamic acid, these results suggested that the hydrolysis of *Y* yielded the amino acid as a racemic mixture, and the hydrolysate was accordingly analysed (Table 2) for DL-glutamic acid using the method of Cohen (1939). After hydrolysis with 5N-NaOH at 37° for 2 hr. the system contained 2 moles NH_3/mole histidine decomposed, and of these, 1 mole appeared to arise by hydrolysis of an alkali-labile bond in compound *Y*: hydrolysis with 4N-NaOH at 100° for 3 hr. yielded in addition approximately 1 mole each of DL-glutamic acid, formic acid and amino nitrogen. Since no racemization occurred when L-glutamic acid was heated for 3 hr. at 100° with 4N-NaOH in a glass vessel, it appeared unlikely that the former results were due to the initial formation of the L isomer and its subsequent racemization. The chemical hydrolysis of compound *Y* therefore proceeds stoicheiometrically according to the equation

$$Y \rightarrow \text{DL-glutamic acid} + HCOOH + NH_3.$$

Edlbacher & Kraus (1930) had previously described a substance which was formed during the degradation of histidine by liver extracts and whose properties were analogous to those of compound *Y*. But whereas in our experiments alkaline hydrolysis yielded racemic glutamic acid, the German workers reported only the formation of the L isomer.

Isolation of glyoxaline compound X. A sample of acetone powder (1·4 g.) was incubated at 37° with L-histidine (374 mg. in 55 ml. water) and 0·1M-Na_2CO_3–$NaHCO_3$ buffer (pH 9·5, 160 ml.) under a gas phase of O_2-free N_2. On the basis of the results of the preliminary experiments, the reaction was allowed to proceed until 1 mole of NH_3/mole of histidine added had been formed, samples being withdrawn periodically and analysed for NH_3 by the procedure of Parnas & Heller (1924). During these determinations, the bulk experimental solution was cooled and stored in the refrigerator. After a total incubation period of 55 min., the amount of free NH_3 indicated that all the histidine had been decomposed. The experimental system was brought to pH 4 with 2N-HCl, 10 ml. 40% (w/v) trichloroacetic acid added and the precipitated proteins removed by centrifuging. The precipitate was washed free from soluble diazo-reacting glyoxaline material and the supernatant and washings were combined and evaporated to a small volume under reduced pressure at 50°. Trichloroacetic acid was removed from the concentrated solution by extracting with ether for 2 hr. in a Kutscher-Steudel extractor, and then the picrate of the glyoxaline compound was precipitated by the addition of a warm saturated solution of picric acid in ethanol. Recrystallization of the picrate from water yielded 114 mg. of yellow needles, m.p. 220°. The picrate was converted into the free base by treating a warm aqueous solution with a solution of tri-*n*-octylamine (10%, v/v) in $CHCl_3$ (cf. Hughes & Williamson, 1951). After removal of the $CHCl_3$ layer, the aqueous layer was concentrated over a boiling-water bath until crystals began to separate. Recrystallization from hot water yielded compound *X* in the form of colourless iridescent needles, m.p. 216–218°.

Identification of glyoxaline compound X as urocanic acid. The ultraviolet absorption spectrum of histidine and of the isolated compound *X* in water at pH 7·2 were very different. The spectrum of the latter contained a marked absorption peak at 273 mμ. and was characteristic of an unsaturated compound containing a conjugated carbon-to-carbon double-bond system (Fig. 2*a*). Compound *X*, but not histidine, was reduced by H_2 in the presence of colloidal Pd,

Table 2. *Products obtained by alkaline hydrolysis of the compound Y formed from histidine by the prolonged action of the acetone powder*

Complete system: 0·05M Sørensen phosphate buffer (pH 7·2), 295 mg. acetone powder and 223 μmoles histidine; total volume, 10 ml. Control system: no histidine. Gas phase, O_2-free N_2; temp., 37°. Incubation period, 2·5 hr. NH_3 estimated by K_2CO_3–$NaBO_2$ (mild alkali) and by 5N-NaOH (strong alkali) in Conway unit. Values in control subtracted from those in complete system.

	mole/mole histidine decomposed			
Product	Unhydrolysed	5N-NaOH 2 hr., 37°	5N-NaOH 16 hr., 37°	4N-NaOH 3 hr., 100°
Glyoxaline	0·0	0·0	0·0	0·0
Formic acid	0·0	0·0	0·6	0·9
Amino nitrogen	0·0	0·0	0·6	0·9
DL-Glutamic acid	0·0	—	—	0·9
L-Glutamic acid	0·0	0·0	0·3	0·5
Free NH_3 (mild alkali)	1·2	2·0	2·0	2·0
Total NH_3 (strong alkali)	2·0	2·0	2·0	2·0

and the product did not possess this absorption peak. Assuming that the glyoxaline compound contained one reducible carbon-to-carbon double bond, microhydrogenation would lead to the uptake of 1 mole H_2/mole compound *X*, and by following the reduction in manometers, the molecular weight was calculated to be 171 if the substance had been dried *in vacuo* over P_2O_5 for 15 hr. at 20° and 138 if dried in an oven for 12 hr. at 120°. The difference (33) between these two values indicates that compound *X* lost two molecules of water of crystallization on prolonged heating. The molecular weight of urocanic acid is 138 and, according to Edlbacher & von Bidder (1942), this acid crystallizes as the dihydrate, from which water of crystallization is lost with difficulty and, in their experiments, only on heating at 100° for 3 hr. over conc. H_2SO_4.

The method of Cunningham, Dawson & Spring (1951) was used to determine the molecular weight of the picrate of compound *X* which had been dried *in vacuo* over P_2O_5 at 78° for 7 hr. and then at 21° for 12 hr. A solution of the picrate (1·466 g./l.) gave a value of $\log_{10} I_0/I$ at 380 mμ. of 0·516. Assuming that 1 mole of picric acid combined with 1 mole of glyoxaline compound, the molecular weight of the latter was calculated to be 152. If the isolated compound was urocanic acid (mol.wt. 138), it was concluded that the picrate crystallized with 1 mole of water which was not removed under the drying conditions described. After drying at 120° for 12 hr., compound *X* gave C, 52·0; H, 4·29; N, 20·1. Calc. for $C_6H_6N_2O_2$: C, 52·2; H, 4·3; N, 20·3%. The mixed m.p. of the isolated compound (m.p. 216–218°) with urocanic acid (m.p. 216–217°) prepared according to Edlbacher & von Bidder (1942) was 216–218°. Edlbacher & Heitz (1942) regard the urocanic acid produced from histidine by the method of Edlbacher & von Bidder (1942) to be the *trans* isomer, the *cis* isomer having a lower m.p. (176°) and crystallizing in the form of rosettes. Levy (1935) has shown that, in the titration curve of histidine, parts 1–3 represent the titration of the carboxyl, glyoxaline and α-amino groups respectively (Fig. 3). A comparison of this curve with that of compound *X* revealed that the latter did not contain part 3, thus confirming that the isolated glyoxaline compound did not contain an α-amino group.

It was, therefore, concluded that compound *X* was urocanic acid and this was further supported by qualitative chemical reactions. When treated with glacial acetic acid, the compound first dissolved and then precipitated as opaque white needles, a reaction characteristic of urocanic acid (Siegfried, 1898). Like urocanic acid, aqueous solutions of compound *X* yielded insoluble derivatives when treated with aqueous phosphotungstic acid, aqueous $AgNO_3$, $HgSO_4$ in 2N-H_2SO_4 or aqueous flavianic acid (2:4-dinitro-1-naphthol-7-sulphonic acid).

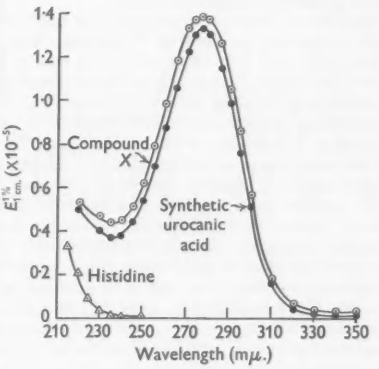

Fig. 2*a*.

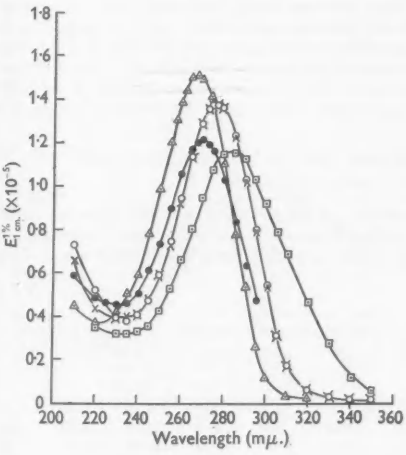

Fig. 2*b*.

Fig. 2. Absorption spectra. (*a*) Compound *X*, synthetic urocanic acid and histidine in aqueous solution at pH 7. (*b*) Synthetic urocanic acid: ●, in distilled water with no additions, pH 6·1; ○, in distilled water brought to pH 7·2 with NaOH; ×, in Sørensen phosphate buffer (0·01 M, pH 7·2); △, in dilute HCl (pH 1·6); ⊡, in dilute NaOH (pH 13·5).

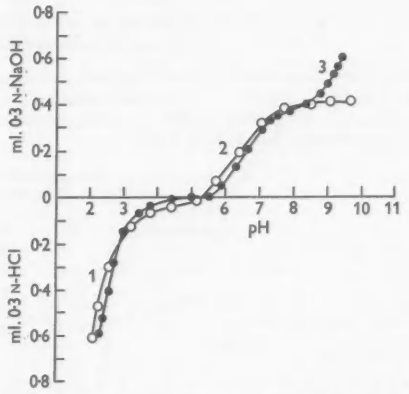

Fig. 3. Titration curve of compound *X* (○) and histidine (●) at 21°. Compound *X* (20 mg.) or histidine (22·5 mg.) in 10 ml. water titrated with NaOH or HCl (0·3N) from an Agla micrometer syringe (Burroughs, Wellcome and Co. Ltd., London).

The absorption spectrum and spectrophotometric determination of urocanic acid. When urocanic acid (prepared according to Edlbacher & von Bidder, 1942) is dissolved in distilled water and the pH brought to 7·0 by the addition of 0·1 N-NaOH, the absorption peak is at 275–277 mμ. and $E^{1\%}_{1cm.}$ max. is $1{\cdot}38 \times 10^5$ (Fig. 2*b*). As is to be expected with an amphoteric substance, the position of the absorption peak depended on the pH of the system. As the pH was decreased, the peak shifted towards 265 mμ. (in 0·1 N-HCl). In alkaline conditions, the peak shifted towards 285 mμ. (in 0·1 N-NaOH). But though the position of the absorption peak altered, there was no appreciable variation of the $E^{1\%}_{1cm.}$ max. value in the pH range 3·3–10·5 (urocanic acid in distilled water plus HCl or NaOH as required), except that, when dissolved in distilled water and in the absence of any addition of acid, alkali or buffer, the maximum absorption was always lower, and was found to be, for example, $1{\cdot}19 \times 10^5$ at pH 6·4 with the peak at 270 mμ. Though phosphate ions have been reported to increase the absorption of solutions of urocanic acid (Hall, 1952), the presence of 0·01 M to 0·1 M Sørensen phosphate buffer (pH 7·2) had no observable effect on the ultraviolet absorption spectrum, and this observation agrees with those of Mehler & Tabor (1953), who give $E^{1\%}_{1cm.}$ max. $1{\cdot}45 \times 10^5$ for urocanic acid in 0·01 M potassium phosphate buffer (pH 7·4). The absorption of urocanic acid solutions is constant for 24–48 hr. and then, even though they are kept in the dark, the absorption begins to decline, as noted by Hall (1952). However, in contrast to his results, the addition of phosphate ions in the present work did not retard this decrease.

Hence, urocanic acid can be estimated satisfactorily by ultraviolet spectrophotometry provided the solutions containing it are assayed within 24 hr. and their pH is adjusted to a predetermined value. The latter is most conveniently done by the addition of a buffer, e.g. an equal volume of 0·2 M Sørensen phosphate buffer, pH 7·2. Under these conditions, there is a linear relationship between extinction at 276 mμ. and concentration in the range 1–10 μg./ml.

The absorption characteristics of compound *X* were identical with those of urocanic acid in all respects, and when dissolved in water and the pH brought to 7·0 by the addition of KOH, the absorption peak was at 276 mμ. with $E^{1\%}_{1cm.}$ max. $1{\cdot}40 \times 10^5$.

Fermentation of urocanic acid. If urocanic acid is an intermediate in the fermentation of histidine, then it is to be expected that, provided the cells are not impermeable, it should be fermented by washed-cell suspensions of *Cl. tetanomorphum*. From Table 3, it can be seen that the fermentation of the isolated urocanic acid (compound *X*) and of synthetic urocanic acid yielded the same products and, with the exception of 1 mole of NH_3, the same proportions of products as the fermentation of histidine.

Preparation of histidine α-deaminase in cell-free state. Samples of acetone powders of *Cl. tetanomorphum* were incubated at 37° for 2·5, 4·5 or 15 hr. with various buffer solutions (final concn., 30 mg. powder/ml.) and at the end of the incubation period, the insoluble material was removed by centrifuging and the supernatant tested for the ability to deaminate histidine to urocanic acid. Extraction with 0·1 M Sørensen phosphate buffer (pH 7·0) resulted in preparations which contained the deaminase and other enzyme systems, since histidine was degraded with the release of 1 mole of free NH_3 and the production of a non-glyoxaline compound which when treated with strong alkali yielded 1 mole of alkali-labile NH_3. On the other hand, extraction for 15 hr. with 0·01 M sodium acetate buffer (pH 4·5) containing 1% (w/v) KCl yielded a clear supernatant which appeared to contain only the histidine α-deaminase system in an active state, and this was the procedure used to obtain enzyme preparations for the subsequent kinetic experiments. The formation of NH_3 and of urocanic acid ran parallel to one another (Fig. 4), the optimum pH being in the region 8·3–9·0 (Fig. 5). The effect of substrate concentration was studied by incubating the enzyme preparation with different concentrations of histidine at pH 9. The histidine concentrations are plotted in Fig. 6 as the logarithms of the arithmetic means of the

Table 3. *Products formed by fermentation of histidine, compound X and urocanic acid by washed-cell suspensions of* Cl. tetanomorphum

Complete system contained 0·04 M Sørensen phosphate buffer (pH 7·4), 13 mg. dry wt. of cells and 10 μmoles histidine (*H*) or 1·38 mg. compound *X* (= 10 μmoles, assuming it is urocanic acid) or 10 μmoles urocanic acid (*U*). Gas phase, O_2-free N_2; temp., 37°. NH_3 determined by Parnas technique (*a*), and by Conway procedure, using NaOH (final concn. 5 N) (*b*). Other experimental details as in Table 1.

	μmoles							μmoles product/μmoles substrate		
		Complete system			Product due to substrate					
Products	Control no substrate (A)	*H*	*X* (B)	*U*	*H*	*X* (B − A)	*U*	*H*	*X*	*U*
H_2	4·2	5·6	8·2	7·9	1·4	4·0	5·7	0·14	0·40	0·57
CO_2	4·9	14·4	14·7	14·2	9·5	9·8	9·3	0·95	0·98	0·93
NH_3 (*a*)	9·1	37·0	26·4	26·3	27·9	17·3	17·2	2·79	1·73	1·72
(*b*)	16·3	44·3	33·8	33·8	28·0	17·5	17·5	2·80	1·75	1·75
Volatile fatty acids										
Total in soln. *A*	22·5	47·7	47·1	48·8	25·2	24·6	26·3	2·52	2·46	2·63
Formic acid	2·1	11·4	11·5	11·9	9·3	9·4	9·8	0·93	0·94	0·98
Total in soln. *B*	20·1	36·0	35·2	35·9	15·9	15·1	15·7	1·59	1·51	1·57
Acetic acid	—	—	—	—	11·8	11·3	11·8	1·18	1·12	1·18
Butyric acid	—	—	—	—	4·1	4·2	3·9	0·41	0·39	0·39
% C recovery	—	—	—	—	—	—	—	98	95	97
% N recovery	—	—	—	—	—	—	—	93	88	88

molarity of the histidine added initially and of the histidine remaining at the end of the 15 min. incubation period. The latter was calculated from the figures obtained for NH_3, on the basis that 1 mole of NH_3 arose from 1 mole of histidine. This procedure was justified by the fact that no alkali-labile NH_3 was detected, indicating that histidine was degraded only as far as urocanic acid. In the experimental conditions used, the activity of the histidine α-deaminase was inhibited by substrate concentrations greater than 0·01 M. This result is to be contrasted with that of Walker & Schmidt (1944), who used a purified liver 'histidase' preparation and observed no inhibition even with histidine concentrations greater than 0·035 M.

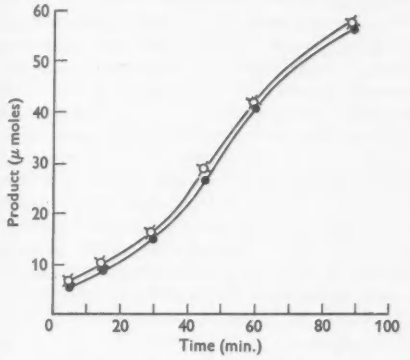

Fig. 4. Progress curve of histidine decomposition by a cell-free preparation of histidine α-deaminase. Complete system contained 0·75 ml. extract of acetone powder with 0·04 M-Na_2CO_3–$NaHCO_3$ buffer (pH 9·0), 89·2 μmoles histidine; total volume 2·5 ml. Control systems contained no histidine. Temp. 37°. Incubated anaerobically in Warburg manometers and reaction stopped at time indicated by addition of acid. ○, NH_3 by Parnas procedure; ●, urocanic acid by ultraviolet spectrophotometric analysis; ×, histidine decomposed (histidine added – residual histidine as determined by decarboxylase). Values in control systems subtracted in all cases.

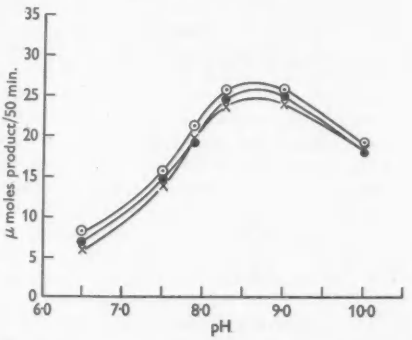

Fig. 5. Effect of pH on activity of histidine α-deaminase. Complete systems contained 0·04 M-$NaHCO_3$–Na_2CO_3 or $NaHCO_3$–CO_2 as required for desired pH, 1 ml. extract of acetone powder, 44·6 μmoles histidine; total volume 2·5 ml. Control systems contained no histidine. Temp. 37°. Incubated anaerobically for 50 min.; reaction stopped by addition of acid. NH_3 (●), urocanic acid (×) and histidine decomposed (⊙) estimated as in Fig. 4. Values in control systems subtracted in all cases.

DISCUSSION

On the basis of the results presented in this paper, it is concluded that the first step in the fermentation of histidine by *Cl. tetanomorphum* is deamination of the amino acid to urocanic acid, and that glutamic acid is an intermediate in the conversion of the latter into hydrogen, carbon dioxide and fatty acids. The initial deamination reaction is analogous to the conversion of aspartic acid into fumaric acid by aspartase. Furthermore, urocanic acid is now known to be an intermediate in the catabolism of histidine in mammalian liver (Sera & Yada, 1939; Takeuchi, 1941; Hall, 1952), and also in other micro-organisms, e.g. *Ps. fluorescens* (Tabor, Mehler, Hayaishi & White, 1952), *Aerobacter aerogenes* (Magasanik, 1953) and certain strains of *Esch. coli*, *Salmonella* spp. and *Shigella paradysenteriae* (Raistrick, 1919).

Some years previously, during his pioneer work on the catabolism of histidine, Edlbacher had already considered the possibility that urocanic acid was an intermediate, but though this substance

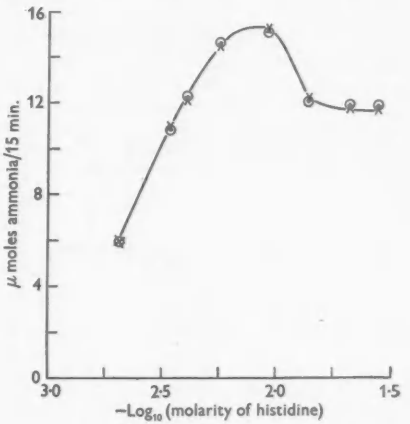

Fig. 6. Effect of substrate concentration on activity of histidine α-deaminase. Complete systems contained 0·04 M-Na_2CO_3–$NaHCO_3$ buffer (pH 9·0), 0·75 ml. extract of acetone powder and histidine (5·9, 13·9, 17·8, 22·3, 31·2, 44·6, 62·4 and 80·3 μmoles as required); total volume, 2·5 ml. Control systems contained no histidine. Temp. 37°. Incubated anaerobically for 15 min. and reaction stopped by addition of acid. NH_3 (⊙) and urocanic acid (×) estimated as in Fig. 4. Values in control systems subtracted in all cases.

was metabolized by preparations of mammalian liver, Edlbacher & Neber (1934) eventually concluded that the pathways of histidine and urocanic acid catabolism were distinct and effected by two different enzyme systems ('histidase' and 'urocanase'). They regarded their results as indicating that the first step in the decomposition of histidine is the opening of the glyoxaline ring, rather than removal of the α-amino group. However, the evidence was not conclusive, and this belief was only one of the possible interpretations of their results. Recently, it has once more been doubted whether urocanic acid is an intermediate in histidine metabolism, at least in animals (Celander & Berg, 1953). Since this conclusion was based on growth experiments in which rats received urocanic acid instead of histidine, the evidence is indirect, and whilst not giving any information about the breakdown of histidine, may indicate that urocanic acid is not an intermediate in the synthesis of this amino acid.

Assuming that urocanic acid is an intermediate, the steps in its conversion into glutamic acid, and the decomposition of glutamic acid itself, are far from being known or understood. It has been shown above that acetone powders of *Cl. tetanomorphum* convert urocanic acid into a compound *Y*, which on chemical hydrolysis yields 1 mole each of DL-glutamic acid, formic acid and ammonia. Though compound *Y* has not yet been isolated and identified, a substance with properties akin to compound *Y* was discovered by Edlbacher during his original work with liver, and substances with similar or identical properties have been isolated from the action of mammalian liver systems on histidine (Tabor, Silverman, Mehler, Daft & Bauer, 1953; Borek & Waelsch, 1953) and from the urine of folic acid-deficient animals (Silverman, Gardiner & Bakerman, 1952). The products formed by the chemical hydrolysis of such compounds have led to the suggestions that formyl-L-isoglutamine (Sera & Aihara, 1942; Oyamada, 1944) or α-for-

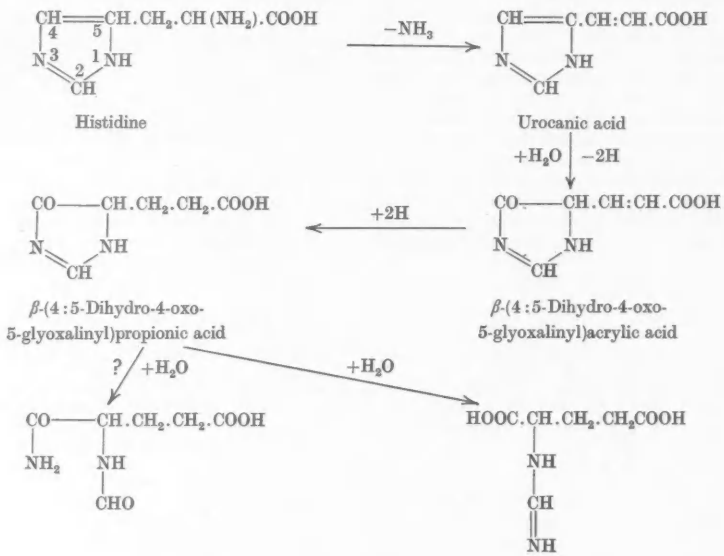

Fig. 7. A tentative scheme for the conversion of histidine into glutamic acid.

mimidoylaminoglutaric acid (L-formamidinoglutaric acid) (Walker & Schmidt, 1944) is an intermediary in the formation of glutamic acid from histidine or urocanic acid. Borek & Waelsch (1953) have presented evidence that the substance they isolated is α-formimidoylaminoglutaric acid. If this is so, the problem arises as to the way in which C-4 of urocanic acid is oxidized to the —CO— group in the α-carboxyl group of glutamic acid. Though there are no experimental data concerning this step, it is possible, in view of the somewhat similar chemical properties of glyoxaline, pyrimidine and purine ring systems, that this oxidation is brought about by an oxidase analogous to xanthine oxidase. The oxidation may be thought of as the addition of the elements of water to the glyoxaline ring, OH to C-4 and H to N-3, followed by the removal of 2H from the C-4 position. In this case the product would be L-β-(4:5-dihydro-4-oxo-5-glyoxalinyl)acrylic acid and the hydrogen removed in the oxidation may eventually be used for reduction of the double bond in the side chain, thus forming L-β-(4:5-dihydro-4-oxo-5-glyoxalinyl)propionic acid. Oyamada (1944) has also suggested that the latter is an intermediate in histidine catabolism.

Whether in fact compound *Y* still contains an unbroken heterocyclic ring is not yet known, but some idea of its possible structure can be obtained by considering what is known about the chemical cleavage of other compounds containing the same skeleton as the glyoxaline nucleus. Dakin (1910) has shown that the mild hydrolysis of optically active hydantoins by alkali yielded carbon dioxide, ammonia and the corresponding racemic amino acid, and that a necessary condition for racemization was the presence of a hydrogen atom at the asymmetric carbon atom. Furthermore, the carbon and nitrogen atoms which become the α-carboxyl and α-amino groups of the amino acid should be combined, since no racemization of optically active amino acids, their uramino acids, or optically active dipeptides occurred under the same conditions of alkali treatment. Other observations in support of this conclusion were made by Levene, Steiger & Marker (1931). Since the alkaline hydrolysis of both compound *Y* and hydantoins yields the same types of products, i.e. a racemic amino acid, ammonia and a one-carbon compound, it is feasible that compound *Y* contains a heterocyclic ring and is perhaps an oxoglyoxalinyl compound of the type suggested above.

On the basis of what is now known as a result of work with micro-organisms and mammalian liver, a tentative scheme (Fig. 7) can be put forward as being the route of histidine degradation to glutamic acid.

SUMMARY

1. Washed-cell suspensions of *Cl. tetanomorphum* ferment histidine to hydrogen, carbon dioxide and ammonia, together with formic, acetic and butyric acids.

2. The first step in the fermentation of histidine is deamination and the formation of urocanic acid. The latter was isolated and characterized and, like synthetic urocanic acid, is fermented by washed-cell suspensions of *Cl. tetanomorphum*.

3. The histidine α-deaminase has been prepared in a cell-free state from acetone powders of *Cl. tetanomorphum* and some of its properties studied.

4. The ultraviolet absorption spectrum of urocanic acid is characteristic and, though the position of the peak (in the region of 276 mμ. at pH 7) is dependent on the pH of the solution, the $E^{1\%}_{1\,cm.}$ max. value shows little change in the pH range 4–10. Phosphate has no effect on the absorption spectrum. Urocanic acid can be assayed by ultraviolet spectrophotometry.

5. Glutamic acid is an intermediate in the fermentation of histidine by *Cl. tetanomorphum*.

6. A pathway is suggested for the conversion of histidine into glutamic acid.

We wish to acknowledge our thanks to Dr S. R. Elsden for his constant interest and advice throughout the course of this work.

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Oestrogenic Activity of Subterranean Clover

1. THE OESTROGENIC ACTIVITY OF GENISTEIN

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Disturbances of reproduction in sheep which had been grazed on pastures dominated by an early flowering strain of subterranean clover (*Trifolium subterraneum* L. var. *Dwalganup*), so-called because it buries its seed, were described by Bennetts, Underwood & Shier (1946). The condition is characterized by infertility of the female, by uterine inertia and by prolapse of the uterus, and as a result of these disorders the lambing may fall below 10% and the loss of ewes may reach 30%. The fertility of the male is not impaired, although the male castrate exhibits extensive changes in the uro-genital tract (Bennetts, 1946, 1947), while mammary development and milk secretion are seen both in the male castrate and the virgin female. These effects, together with the constant occurrence in the infertile ewes of cystic hyperplasia of the endometrium, provided strong evidence of prolonged oestrogenic stimulation.

Oestrogenic extracts were prepared from subterranean clover by Curnow, Robinson & Underwood (1948). Robinson (1949) and Beck & Braden (1951) demonstrated the phenolic nature of the oestrogen and reported methods of preparation of purified extracts for use in the biological estimation of the amount of oestrogen in the clover. The oestrogen was later concentrated by precipitating it in the 'chloroplast' fraction of a press-juice of clover (Legg, Curnow & Simpson, 1950), and this was followed by the isolation of small amounts of genistein (5:7:4′-trihydroxy-*iso*flavone) from the chloroplast fraction (Bradbury & White, 1951). In preliminary tests genistein showed an oestrogenic activity of approximately 10^{-5} that of oestrone. Formononetin (7-hydroxy-4′-methoxy-*iso*flavone) was also isolated from the 'chloroplast' fraction but was inactive.

Since genistein is a new type of natural oestrogen it is of interest to examine its biological properties and activity in detail. The investigations to be reported below have been carried out in two centres, Sydney, N.S.W., and Perth, W.A., and consist of detailed studies of the compound by means of the Allen Doisy vaginal cornification tests as described by Emmens (1950*a*).

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EXPERIMENTAL

Materials

Genistein. Crystalline genistein, m.p. 301° (decomp.) (corr.) was used. The samples used in Sydney were synthetic and prepared by Mr R. B. Bradbury. In Perth two samples were used, one synthetic and the other a sample prepared from subterranean clover (Curnow, 1954). Mixed melting

points of the synthetic and natural compounds showed no depression and no differences were observed in their oestrogenic activities.

Formononetin. Crystalline formononetin, m.p. 258° (corr.) prepared from clover was used.

Oestradiol-3:17β. This has been used as the reference oestrogen.

Experimental animals and assay procedure

Sydney. Groups of ovariectomized mice were used in the tests described below. The general details of management and randomization of experimental groups have been described previously (Biggers, 1951), and the details of the subcutaneous and intravaginal Allen Doisy techniques of oestrogen assay have been described by Emmens (1950*a*). The oestrogens were injected twice daily (9.30 a.m. and 4.30 p.m. approx.) for 2 days. The designs, results and analyses of the experiments will be described together. Standard methods of probit analysis have been used in the analysis of results (Finney, 1952).

In all the recent work on the intravaginal action of oestrogens aqueous media have been used as solvents, e.g. 50% aqueous glycerol (Emmens, 1950*b*; Biggers, 1951; Sullman, 1952), distilled water (Biggers, 1953*a*) and aqueous protein solutions (Biggers, 1953*b*). Unfortunately, genistein in the dose required is insoluble in all these solvents. Although genistein is soluble in nut oil, this solvent has been found unsatisfactory in intravaginal tests in mice (Emmens, 1939). However, propylene glycol may be substituted for water in the intravaginal method and does not effect the activity of oestrone (Claringbold, in preparation). Since genistein is readily soluble in propylene glycol, this solvent has been used for both the subcutaneous and intravaginal tests. The genistein was suspended in the propylene glycol and dissolved by gentle heating.

A severe limitation to the intravaginal method of assaying oestrogens of very low activity is the small volume of injection which must be used to avoid leakage from the vagina. Biggers & Claringbold (1954) have shown in the albino strain of mice used in Sydney that the maximum permissible volume of each injection is 0·01 ml. In the case of genistein this necessitates a very concentrated solution. These solutions may be prepared by heating and allowing them to cool slowly to body temperature before injection. The solution remains supersaturated for several hours and may be injected easily in this state.

Perth. The methods used were essentially similar to those described above, following the methods described by Emmens (1950*a*). The oestrogens were injected subcutaneously or administered orally by stomach tube, oestradiol-3:17β as a solution, and genistein and formononetin as suspensions in peanut oil. The volume of each injection was 0·05 ml.

In both Sydney and Perth a smear consisting only of nucleated or cornified cells was read as positive and a smear containing leucocytes as negative.

RESULTS

Sydney

The dose/response line (subcutaneous administration). Six dose/response lines were obtained by the subcutaneous injection of genistein in propylene glycol using four injections in 2 days. The weighted mean slope of the lines is 6·85, S.E. 0·80, and the χ^2 test shows the six estimates to be homogeneous ($\chi^2_{[5]}=8{\cdot}88$, $0{\cdot}2>P>0{\cdot}1$). The common slope was used to calculate the six individual median effective doses (M.E.D.). The six estimates of the M.E.D. are also homogeneous ($\chi^2_{[5]}=5{\cdot}66$, $0{\cdot}5>P>0{\cdot}3$) and the weighted mean M.E.D. found to be 2·53 mg. (fiducial limits of error ($P=0{\cdot}05$), 2·35 to 2·73). Thus the data do not provide evidence of secular changes in the sensitivity of the mice to the genistein such as have been found with highly active oestrogens (Emmens, 1939).

The dose/response line (intravaginal administration). Three dose/response lines were obtained by the intravaginal injection of genistein in propylene glycol using four injections in 2 days. The weighted mean slope is 0·91, S.E. 0·32, and the χ^2 test shows the three estimates to be a homogeneous group ($\chi^2_{[2]}=1{\cdot}789$, $0{\cdot}5>P>0{\cdot}3$). The common slope was used to compute the individual M.E.D.'s but in this case they form a heterogeneous group ($\chi^2_{[2]}=9{\cdot}38$, $0{\cdot}01>P>0{\cdot}001$). The shifts in sensitivity are similar to those reported for the intravaginal dose/response line to oestrone (Biggers, 1953*a*).

It can be seen that the slope of the intravaginal dose/response line is considerably less than the slope of the subcutaneous dose/response line.

The relative activity of genistein. Three tests were carried out to determine first the relative potency of genistein in terms of oestradiol-3:17β, and secondly the ratio of the M.E.D. for subcutaneous administration to the M.E.D. for intravaginal administration (S/L ratio, see Emmens, 1941). The dose/response lines within each test were determined simultaneously to overcome the possibility of secular shifts in sensitivity. The results are shown in Table 1.

(i) *Relative potency.* Data in Tests 1 and 2 permit the estimation of the relative potency of genistein in terms of oestradiol-3:17β when both are given in propylene glycol. Table 2 shows the partitioning of χ^2. The component of χ^2 for heterogeneity is just significant at the 5% level. However, in a situation where there are small expectations (as in this case) the usual method of calculating the heterogeneity χ^2 may lead to unduly large contributions to χ^2 (Finney, 1952). However, on computing the χ^2 by the 'longhand' method (Emmens, 1948) the χ^2 for heterogeneity is not significant ($\chi^2_{[3]}=5{\cdot}35$, $0{\cdot}2>P>0{\cdot}1$). In view of this finding the dose response lines are assumed to be homogeneous. Table 2 shows that all four lines are parallel, and therefore a common slope and its variance were computed for the estimation of relative potency. The value of $b_c/(s_b)_c$ is 6·74 and the limits of error are computed by the approximate method. These limits should be only slightly narrower than the fiducial

Table 1. *Dose/response data for tests of the subcutaneous and intravaginal action of genistein and oestradiol-3:17β given by four injections in 2 days in propylene glycol*

The dose/response lines in each test were determined simultaneously. The response is shown as the number of animals positive to the number of animals in the group.

	Subcutaneous administration				Intravaginal administration			
	Genistein		Oestradiol-3:17β		Genistein		Oestradiol-3:17β	
Test no.	Dose (mg.)	Response	Dose (mμg.)	Response	Dose (mg.)	Response	Dose (mμg.)	Response
1	0·25	1:11	14	0:12	—	—	—	—
	0·5	0:12	19	1:12	—	—	—	—
	1·0	0:12	25	0:12	—	—	—	—
	2·0	2:12	34	2:11	—	—	—	—
	4·0	11:12	46	8:12	—	—	—	—
2	1·44	0:11	32	6:12	0·53	5:12	0·05	1:12
	1·80	0:11	40	6:12	0·80	3:12	0·1	3:12
	2·25	5:10	50	11:12	1·20	5:12	0·2	7:12
	2·81	7:11	62·5	9:11	1·80	6:12	0·4	9:12
	3·51	8:12	78·1	11:12	2·70	7:12	0·8	11:12
3	1·44	0:12	—	—	0·67	11:36	—	—
	1·80	2:12	—	—	1·00	12:36	—	—
	2·25	5:12	—	—	1·50	22:36	—	—
	2·81	6:12	—	—	2·25	18:36	—	—
	3·51	12:12	—	—	3·38	18:36	—	—

Table 2. *Partitioning of χ^2 for the two dose/response lines obtained with genistein and the two dose/response lines obtained with oestradiol-3:17β by using subcutaneous administration in Tests 1 and 2*

Source of variation	Degrees of freedom	χ^2	P
Parallelism	3	2·94	0·5–0·3
Heterogeneity	5	12·90	0·05–0·02

Table 3. *Partitioning of χ^2 for the dose/response lines obtained with genistein by both subcutaneous and intravaginal administration in Tests 2 and 3*

Source of variation	Degrees of freedom	χ^2	P
Parallelism:			
Between mean slopes	1	21·48	0·001
Within mean slopes	2	0·47	0·8–0·7
Heterogeneity	8	15·70	0·05–0·02

limits of error (Irwin, 1943). The relative potency of genistein in terms of oestradiol-3:17β is found to be $1{\cdot}25 \times 10^{-5}$, limits of error ($P = 0{\cdot}05$) $1{\cdot}03 \times 10^{-5}$ to $1{\cdot}51 \times 10^{-5}$ or 83–121 %.

(ii) *S/L ratio.* The data of tests 2 and 3 permit the estimation of the S/L ratio for genistein. Also the data of test 2 allow a similar estimation to be made for oestradiol-3:17β for comparison. Table 3 shows the partitioning of χ^2 for the two lines obtained by subcutaneous administration and the two lines obtained by intravaginal administration. Again the heterogeneity χ^2 is just significant, but as the χ^2 obtained by the 'longhand' method is not significant ($\chi^2_{[8]} = 9{\cdot}85$, $0{\cdot}3 > P > 0{\cdot}2$), homogeneity of the lines is assumed. Table 3 shows that while the within mean slope differences are not significant, there is a highly significant difference between the slopes obtained by the subcutaneous and intravaginal methods. Mean slopes were therefore computed from the data obtained by each route of administration and these used to estimate the appropriate M.E.D.'s.

The S/L ratio was computed from the weighted mean M.E.D.'s for each method of administration. The S/L ratio is 1·22, fiducial limits of error ($P = 0{\cdot}05$), 0·73 to 2·05.

Similar computations with the data for oestradiol-3:17β give a value of 175, fiducial limits of error, 108 to 284. Thus genistein is characterized by a very low S/L ratio and falls into the class of pro-oestrogens as defined by Emmens (1941).

Perth

Two tests were made, the first being a comparison of the activity of genistein and oestradiol-3:17β administered subcutaneously, and the second being a determination of the dose/response line obtained with genistein given orally, the two tests being done at different times. The results are given collectively in Table 4.

The relative potency of genistein in terms of oestradiol-3:17β may be calculated from the data. The χ^2 for heterogeneity of the two lines is not significant ($\chi^2_{[1]} = 0{\cdot}65$, $0{\cdot}8 > P > 0{\cdot}7$) nor is the χ^2 for parallelism ($\chi^2_{[2]} = 3{\cdot}45$, $0{\cdot}1 > P > 0{\cdot}05$). Consequently a mean slope was computed for the estimation of relative potency and its exact fiducial limits of error. This was found to be $4{\cdot}53 \times 10^{-5}$, fiducial limits of error ($P = 0{\cdot}05$) $3{\cdot}30 \times 10^{-5}$ to $5{\cdot}99 \times 10^{-5}$, or 73–132 %.

Table 4. *Dose/response data for tests of the subcutaneous and oral administration of genistein and the subcutaneous administration of oestradiol-3:17β given by four injections in 2 days*

The data obtained by subcutaneous administration were obtained simultaneously, while the data on the oral administration were obtained separately. The response is shown as the number of animals positive to the number of animals in the group.

Genistein				Oestradiol-3:17β	
Subcutaneous		Oral		Subcutaneous	
Dose (mg.)	Response	Dose (mg.)	Response	Dose (mμg.)	Response
0·25	2:20	2·5	0:20	20	3:20
0·50	7:20	5·0	3:20	30	13:20
1·00	16:20	10·0	12:20	45	18:20
		15·0	19:20		
		20·0	20:20		

This estimate of the relative potency is significantly different ($P > 0.001$) from the estimate obtained in Sydney, being about 3·5 times as great. This is probably due to differences in technique, such as the solvent employed as the vehicle of administration. It is well known that such factors can have a profound influence on the results (Emmens, 1939; Pedersen-Bjergaard, 1939). However, the results from the two centres clearly indicate that genistein has very low oestrogenic activity.

The slope of the dose/response line obtained by the oral administration of genistein is 5·65, S.E. 0·95. The M.E.D. was computed and found to be 8·20 mg.; fiducial limits of error ($P = 0.05$) 6·74–9·97, or 82–122 %.

Formononetin, injected at a level of 10 mg. or given orally at 50 mg., elicited no vaginal response.

DISCUSSION

Although observations of oestrogenic activity in plant material have been made frequently since the original work of Loewe (1926), who induced oestrus in ovariectomized mice with ether extracts of willow catkins and of ovaries of the water lily (*Nuphar luteum*), very few oestrogenic compounds have been crystallized from plant material. Butenandt (1932) and Butenandt & Jacobi (1933) were the first workers to crystallize and identify an oestrogen of plant origin. Their starting material was the residue from the pressure extraction of oil obtained from palm kernels. This yielded a crystalline product identical with oestrone. Skarzynski (1933*a*, *b*) reported the isolation of oestriol from female willow flowers. A highly active oestrogen was isolated from the tubers of *Butea superba*, a leguminous vine common in China and Thailand, by Schoeller, Dohrn & Hohlweg (1938, 1940). The activity was about twice that of oestrone when injected subcutaneously in rats. Butenandt (1940) described further work on this substance and gave it a formula of $C_{19}H_{22}O_6$, although the structure of the oestrogen has not yet been elucidated.

Emmens (1941) compared the median effective doses of an oestrogen when administered by the subcutaneous and intravaginal routes (S/L ratio). Several compounds were examined and two groups of substances were distinguished, one where the S/L ratio is near unity and the other where the ratio is of the order of hundreds. The substances in the first group are called pro-oestrogens and those in the second true oestrogens. All the natural oestrogens found in animals belong to the group of true oestrogens. Later, Emmens (1942) examined these substances in mice in which the vagina had been surgically divided to give separate vaginal pouches. He found that a dose of a true oestrogen which was just sufficient to cornify the epithelium of one pouch when placed on it failed to produce cornification in the second pouch. However an effective dose of a pro-oestrogen for one pouch caused cornification in both pouches. It is believed that true oestrogens act directly on the cells of the responding epithelium, whereas the pro-oestrogens are absorbed into the body and give rise to true oestrogenic substances during their metabolism.

The results presented above show that genistein is a pro-oestrogen, and presumably gives rise to oestrogenic products during its metabolism in the body. The estimates of relative potency show that genistein is of very low activity, and this suggests that its oestrogenic properties are caused by only a minor metabolite.

When an oestrogen of such low activity as genistein is isolated from natural sources, there is the danger that the oestrogenic activity is due to a contaminant and not due to the compound under examination. The amount of active substance required for such a situation to arise is of the order of the tolerance limits for impurities which are normally accepted in analytical reagents. There are two reasons, however, for accepting that genistein has oestrogenic properties: (1) the synthetic and natural materials were found to be equally active, and it is highly improbable that an active contaminant could be present in equivalent amounts,

(2) the substance has been shown to be a pro-oestrogen, for if the activity were due to a highly active contaminant, the S/L ratio would be expected to be high, the reverse of that actually found.

The results show that the slopes of the regression lines for each route of administration are significantly different. The slope for intravaginal administration is low and indicates that the response is very variable. A possible contributory cause of this variation may be the fact that the highly concentrated propylene glycol solutions used to precipitate in the vagina on contact with aqueous tissue fluids. To what extent this influences the vaginal response awaits further investigation.

SUMMARY

1. The relative potency of genistein in terms of oestradiol-3:17β using four injections given subcutaneously in 2 days, and propylene glycol as solvent, is $1{\cdot}25 \times 10^{-5}$, fiducial limits of error ($P = 0{\cdot}05$) $1{\cdot}03 \times 10^{-5}$ to $1{\cdot}51 \times 10^{-5}$ (Sydney experiments).

2. The relative potency of genistein in terms of oestradiol-3:17β using the same technique, and peanut oil as solvent, is $4{\cdot}53 \times 10^{-5}$, fiducial limits of error ($P = 0{\cdot}05$) $3{\cdot}30 \times 10^{-5}$ to $5{\cdot}99 \times 10^{-5}$ (Perth experiments).

3. Although these two estimates are different, they show that genistein has a very low oestrogenic activity when compared to oestradiol-3:17β.

4. The median effective doses for genistein given orally in four doses in 2 days is 8·20 mg., fiducial limits of error ($P = 0{\cdot}05$) 6·74 to 9·97.

5. Since the S/L ratio of genistein is 1·22, fiducial limits of error ($P = 0{\cdot}05$) 0·73 to 2·05, it is a pro-oestrogen.

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Oestrogenic Activity of Subterranean Clover

2. THE ISOLATION OF GENISTEIN FROM SUBTERRANEAN CLOVER AND METHODS OF QUANTITATIVE ESTIMATION

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The isolation of small amounts of genistein (5:7:4′-trihydroxy-*iso*flavone) from subterranean clover (*Trifolium subterraneum* var. *Dwalganup*) and the demonstration of its weakly oestrogenic properties (Bradbury & White, 1951, and Biggers & Curnow, 1954), together with the difficulties in the synthesis of this compound, made it important that a more efficient method be found for the isolation of this *iso*flavone from the clover. Also, the amount of genistein isolated by Bradbury & White (2 mg./100 g. of fresh clover) and its low oestrogenic activity (Biggers & Curnow, 1954) were not nearly sufficient to account for the activity of the fresh plant (Robinson, 1949) and left considerable doubt as to whether this compound was responsible for the oestrogenic activity of the clover.

The present study has shown, however, that the clover contains much more genistein than found by these workers. The amount now found indicates that genistein could indeed be responsible for the activity of the plant and therefore for the infertility in sheep grazing on subterranean-clover pastures. This finding called for rapid and efficient methods of estimation of this compound in the plant. For the determination of the genistein content of the clover at various stages of growth, under various nutritional conditions, in different seasons and in various strains, an accurate method of estimation was required, whereas for the purposes of the plant geneticist a rapid method was desired for use with small amounts of the material (parts of a single plant), in which great accuracy is a secondary consideration. Methods fulfilling these two sets of requirements have been developed and are presented here.

EXPERIMENTAL

Materials

'Chloroplast fraction'. The 'chloroplast fraction' was prepared as described by Bradbury & White (1951) from the *Dwalganup* strain of subterranean clover. From 4020 kg. of fresh plant 15 kg. of 'chloroplast' was produced.

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Fresh clover. Clover was cut and weighed in the field and placed immediately in ethanol.

Silica gel. Davison Chemical Corporation '200 mesh' silica gel was used.

Bioassay procedure. The methods described in the previous paper (Biggers & Curnow, 1954) were used for the assay of the oestrogenic activity of extracts.

Isolation of genistein from 'chloroplast'

Extraction. An amount of 100 g. of 'chloroplast' was extracted with 1 l. ethanol for 30 min. while being stirred under reflux. The residue was extracted with a further 500 ml. ethanol, under reflux for 15 min. The combined extract contained 7·2 g. of solid. Extraction of the chloroplast residue with ethanol and with ethanolic NaOH failed to yield any further oestrogenic material.

Purification. The combined ethanol extracts were evaporated *in vacuo* to a volume of 600 ml. Water was added to bring the volume to 1000 ml. and the mixture extracted 4 times with 200 ml. benzene. The benzene extracts were washed in countercurrent fashion 4 times with 200 ml. 60% (v/v) ethanol. This treatment removed from the aqueous ethanol all the chlorophyll and fats, and left a clear pale-yellow solution. The dark-green benzene phase contained 2·78 g. of solid which showed no oestrogenic activity on injection in ovariectomized mice either before or after saponification.

The aqueous ethanol phase was evaporated *in vacuo* to a volume of 200 ml. and extracted 4 times with 200 ml. of ether. The aqueous phase after concentration showed no oestrogenic activity. The ether phase was extracted 4 times with 20 ml. saturated aqueous $NaHCO_3$ and then twice with 20 ml. water. (The dark brown aqueous extract was acidified with H_2SO_4 and extraction with ether yielded 0·74 g. of material with no oestrogenic activity.) The ether phase contained 3·50 g. of light yellow solid with oestrogenic activity equal to that of approx. 6 μg. oestradiol. Chromatography of 250 mg. of this material on 50 g. silica gel, by using light petroleum–ether mixtures and determining the ultraviolet absorption curves of the eluates to locate the genistein fractions, as in the macro-method below, yielded 30 mg. of genistein, which after recrystallization from aqueous ethanol gave m.p. 300–301° (decomp.) (corr.) not depressed by a synthetic sample. Thus 100 g. of 'chloroplast' yielded 420 mg. of genistein. Related to the original fresh clover this is equivalent to about 2 mg./100 g. (12 mg./100 g. dry matter), which is the figure given by Bradbury & White (1951).

Isolation of genistein from fresh clover

From 100 g. fresh clover (16 g. dry matter), treated precisely as for the 'chloroplast', 59 mg. pure genistein were isolated. This is equivalent to 369 mg. genistein/100 g. dry matter of clover.

Macro-method of estimation of genistein in clover

Sampling. Samples of 30 g. fresh clover were taken from the field and placed immediately in 200 ml. ethanol. A separate sample was taken for drying and estimation of dry-matter content.

Extraction. The fresh clover was refluxed with the 200 ml. ethanol for 15 min., cooled and filtered. The residue was macerated with a further 300 ml. ethanol in a Townson and Mercer (Croydon, Surrey) top-drive macerator and refluxed for 30 min., cooled and filtered. The macerated residue was washed with 50 ml. ethanol and all the ethanol extracts were combined.

Extraction of the residue, either with ethanol or with ethanolic NaOH, failed to yield any extract with oestrogenic activity.

Aqueous ethanol–benzene partition. The combined ethanol extracts were evaporated *in vacuo* to 150 ml. and water was added to bring the volume to 250 ml. The resulting 60% ethanol solution was extracted with three 50 ml. portions of benzene, the separating funnel being gently rotated to avoid the formation of troublesome emulsions. The three benzene extracts were kept apart and extracted in counter-current fashion with five 50 ml. portions of 60% ethanol.

The benzene extract was evaporated to dryness, hydrolysed under reflux with 100 ml. ethanolic N-NaOH and the 'phenol' fraction isolated in the usual way. Injection in ovariectomized mice showed that it contained less than 5% of the total oestrogenic activity of the original extract.

Thus the fats and chlorophylls were retained in the benzene phase whereas the clear, faintly yellow aqueous-ethanol phase contained more than 95% of the oestrogenic material.

The partition coefficient of genistein between benzene and aqueous ethanol was estimated in the following manner. Samples of 0·5 mg. genistein were shaken with 50 ml. aqueous ethanol (50, 60 and 70%, v/v) and 50 ml. benzene and the phases allowed to separate. The temperature was 19°. The aqueous ethanol phases were evaporated to dryness *in vacuo* and the residues dissolved in 50 ml. ethanol. The extinction at 262·5 mμ. was determined with a Beckman spectrophotometer. Similar determinations were made for formononetin (7-hydroxy-4′-methoxy-*iso*flavone) by using the extinction at 249 mμ. The results are given in Table 1.

The absorption curves of genistein and formononetin in pure ethanol are shown in Fig. 1. Over the range 0·1 mg./100 ml. the optical density at 262·5 mμ. of genistein solutions was found to be directly proportional to the concentration.

With the partition ratio of 1·39 for genistein between 60% ethanol and benzene, the theoretical recovery of genistein in the purification procedure is 97·9%. For formononetin the theoretical recovery is 59·3%. Very little formononetin, however, is found in the purified clover extracts. Formononetin is relatively insoluble in ethanol (175 mg./100 ml. at 22°), whereas genistein is readily soluble.

Separation of phenols. The aqueous ethanol solution was evaporated *in vacuo* to a volume of 100 ml., saturated with NaCl and extracted with four 100 ml. portions of ether. The combined ether solution was then extracted with six 25 ml. portions of 0·1 N-NaOH. CO_2 was passed into the combined NaOH extracts until they were saturated, and the phenols were extracted with four 100 ml. portions of ether. The combined ether solution was washed with three 20 ml. portions of water, and evaporated to dryness to yield the phenols.

Chromatography. An amount of silica gel 200 times the weight of the phenols was transferred with stirring into a water-jacketed column 15 mm. in diameter filled with light petroleum (40–60°) and the phenols were chromatographed on this column with 50 ml. portions of dry light petroleum–ether mixtures as eluting solvents, running from pure light petroleum to 10, 20, 30...100% (v/v) ether. Finally acetone and then methanol were run to elute the column completely. The pressure at the top of the column was increased by nitrogen at 50–150 mm. Hg. From 20 to 30 fractions were obtained in this way. These fractions were weighed and the recoveries from a number of columns varied from 100 to 110%, the excess being due presumably to some material eluted from the silica gel.

Table 1. *Partition of genistein and formononetin between aqueous ethanol and benzene*

The partition ratio (concentration in aqueous ethanol/concn. in benzene) was determined using 50 ml. volumes of the two phases and 0·5 mg. of the substance investigated. The concentrations in the extracts were determined by spectrophotometry. Genistein, $E^{1\%}_{1\,cm.}$ 262·5 mμ. (max.) = 1400. Formononetin, $E^{1\%}_{1\,cm.}$ 249 mμ. (max.) = 1000.

	Aqueous phase		
Substance	50% ethanol (v/v)	60% ethanol (v/v)	70% ethanol (v/v)
Genistein	0·975	1·39	2·37
Formononetin	0·185	0·361	0·645

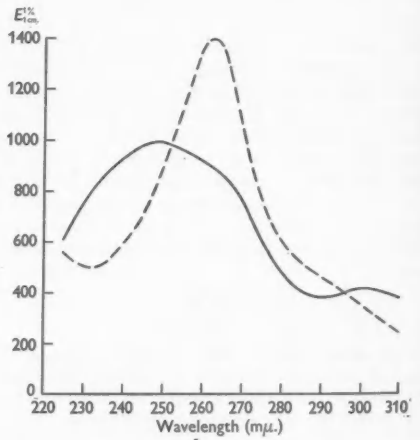

Fig. 1. Absorption curves of genistein (– – –) and formononetin (——) in ethanol solution.

Spectrophotometry. The absorption spectrum, over the range 220–300 mμ., was observed for each fraction in solution in ethanol. A typical series of curves for the fractions is shown in Fig. 2.

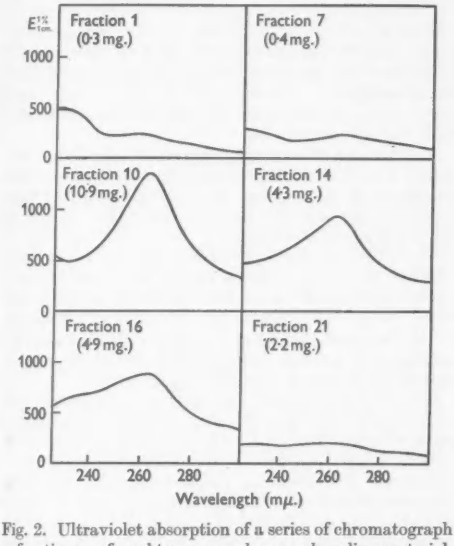

Fig. 2. Ultraviolet absorption of a series of chromatograph fractions of subterranean-clover phenolic material. Fractions 10, 14 and 16 contain genistein; fractions 1, 7 and 21 do not.

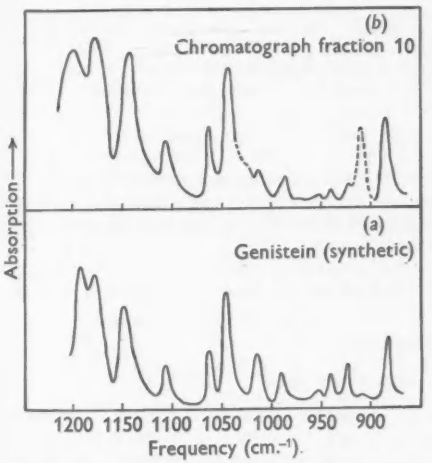

Fig. 3. Infrared absorption curves of (*a*) synthetic genistein and (*b*) chromatograph fraction from subterranean clover. Bands due to impurities in the chromatograph fraction are dotted. (Grubb-Parsons S3A Spectrometer equipped with sodium chloride prism. Solid samples ground with paraffin oil.)

Fractions 9–16 had maxima at 262·5 mμ. and were all similar to the absorption curve of genistein (Fig. 1). On injection in ovariectomized mice, only the fractions 9–16 showed oestrogenic activity, whereas fractions 1–8 and 17–21 were inactive. Recrystallization of fractions 9–16 yielded genistein, identified by its m.p. and u.v. absorption. As a final proof of identity, fraction 10 showed absorption in the infrared region, 860 cm.$^{-1}$ to 1200 cm.$^{-1}$, very similar to that of synthetic genistein, as shown in Fig. 3, with impurities showing absorption at 1030 and 910 cm.$^{-1}$.

In the crude fractions, however, the absorption at the minimum (231 mμ.) was greater than that for pure genistein, whereas the maximum absorption (262·5 mμ.) was low. To compensate for the impurities, an arbitrary line was calculated giving assumed values for the impurities of $E^{1\%}_{1\,cm}$ (231 mμ.) = 450 and $E^{1\%}_{1\,cm.}$ (262·5 mμ.) = 320. If in Fig. 4 the observed values for the fraction are $e = E^{1\%}_{1\,cm.}$ (231 mμ.) and $f = E^{1\%}_{1\,cm.}$ (262·5 mμ.), then

$$500x + 450n = e \quad \text{and} \quad 1400x + 320n = f,$$

where x is the proportion of genistein present and n the 'proportion' of impurities. From these equations:

$$x = (0{\cdot}96f - 0{\cdot}68e) \times 10^{-3},$$

and

$$n = (2{\cdot}98e - 1{\cdot}06f) \times 10^{-3}.$$

To test the validity of the assumed values for the absorption by impurities, x and n were calculated for a number of fractions and the theoretical curve was drawn for the calculated mixture. There was close agreement between the observed and calculated absorption.

The similarity between the absorption by impurities in the genistein fractions and the absorption by the non-genistein fractions suggested that the same formula may be used to estimate genistein from the curves obtained from the

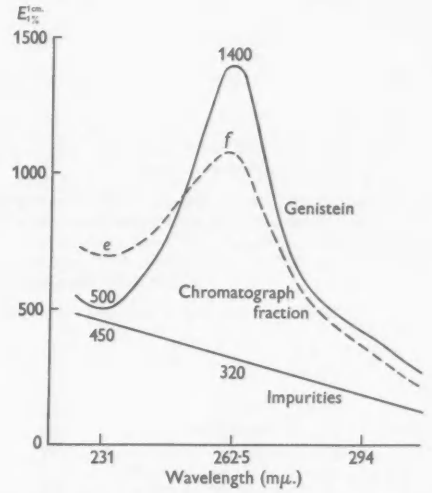

Fig. 4. Arbitrary straight-line absorption assumed due to impurities in chromatograph fractions. From the values shown, the proportion of genistein present in the fraction is calculated as: $x = (0{\cdot}96f - 0{\cdot}68e) \times 10^{-3}$.

whole phenol fraction without chromatography. A typical application of this procedure is demonstrated in Fig. 5, which shows close agreement between the observed and calculated curves.

Thus, by reading the values for $E^{1\%}_{1\,cm.}$ (231 mμ.) and $E^{1\%}_{1\,cm.}$ (262·5 mμ.), the amount of genistein present in the phenols may be calculated. A slide rule was constructed for the purpose of making the calculation. This has been applied to a considerable number of extracts and provides a reasonably accurate method of estimation of genistein in clovers. Similar correction methods have been applied by Morton & Stubbs (1946) in vitamin A determinations and by Sveinsson, Rimington & Barnes (1949) in porphyrin determinations. Genistein added to clover was recovered to the extent of 96–109% in six different estimations.

Micro-method of estimation of genistein in clover

Extraction and purification. Two leaves, of similar age, were taken from each clover plant. Both were weighed, one dried at 100° for the determination of dry matter content, and the other placed in a 3 × ½ in. test tube and refluxed with a cold thimble for 10 min. with 1 ml. ethanol. The ethanol solution was decanted into a second tube graduated at 0·6 and 1·0 ml. The leaf residue was refluxed with a further 0·5 ml. ethanol for 5 min. and the extract decanted into the graduated tube. The combined extracts were evaporated by using a stream of air or nitrogen directed into the tube, keeping the tube warmed to about 70° in a glycerol bath, until the volume was reduced to 0·6 ml. Water was added to bring the volume to 1·0 ml. and 0·5 ml. benzene was added. The tube was rotated at an angle of 45° from vertical in a mechanical rotator at about 140 rev. min.$^{-1}$ for 10 min. This procedure avoided the formation of emulsions and gave an efficient extraction, the benzene layer being dark green in colour whereas the aqueous alcohol layer was almost colourless.

With the use of a pipette inserted to the bottom of the tube, 0·5 ml. of the aqueous phase, free of the benzene phase, was drawn up and a volume equivalent to 2·5 mg. of fresh clover placed in a small tube and evaporated to dryness by the technique described above.

Paper chromatography. Following the method of Bate-Smith & Westall (1950), the extract was dissolved in a minimum of butanol containing 1% HCl (w/v) and transferred with a micro-pipette to a paper strip (Whatman no. 4) 5 mm. wide, care being taken to keep the length of the applied spot within a 5 mm. limit. Control strips were spotted similarly with 2·5 and 5·0 μg. genistein.

The strips were placed in an atmosphere saturated with the vapour of the aqueous phase of a butanol–acetic acid–water mixture (40:10:50, v/v) for 24 hr. The solvent mixture, butanol–acetic acid–water (40:10:20, v/v), was then introduced for ascending chromatography. After the solvent front had moved approx. 30 cm. (18 hr.) the strips were dried in a current of air in the fume hood and sprayed with aqueous 0·1% (w/v) $FeCl_3$ solution, freshly prepared from a 10% stock solution. The pink genistein spot (R_F = 0·9) was compared with the control strips.

Throughout a series of several hundred separations it was found impossible to estimate genistein content by measuring spot length. Also, the separation of genistein from the pigments present was not always complete, so that only a visual approximation of genistein content, by comparing the extract chromatograms with the controls, was possible.

Fig. 5. Observed absorption curve (——) of phenols from subterranean clover (no. P_4) and calculated curve (- - -). For explanation see text.

RESULTS

The results of a series of determinations on different clovers by using silica gel chromatography and ultraviolet absorption measurements are given in Table 2.

Table 2. *The genistein content of several clovers*

30 g. fresh clover were used and the results are calculated on the basis of 100 g. dry matter.

		Ethanol extract					
			60% ethanol phase				
No.	Description	Benzene phase (g.)	Neutrals (g.)	Acids (g.)	Phenols (g.)	Residue (g.)	Genistein (g.)
1	*T. subterraneum* var. *Dwalganup*	10·3	0·5	0·4	1·6	76·4	0·740
2	*T. subterraneum* var. *Burnerang*	11·3	0·5	0·4	1·4	86·6	0·400
3	*T. subterraneum* var. *Red Leaf*	8·2	0·5	0·2	0·9	76·4	0·550
4	*T. subterraneum* var. *Tallarook*	7·7	0·4	0·5	1·2	85·7	0·565
5	*T. repens*	7·9	0·2	0·1	0·2	75·8	Nil
6	*T. fragiferum*	9·4	0·6	0·5	0·2	73·4	Nil
7	*T. pratense*	15·5	0·5	0·1	0·6	76·6	Nil
8	*Melilotus alba*	9·3	0·4	0·3	0·2	71·2	Nil

The four subterranean clovers contained large amounts of genistein, whereas the others contained no trace of genistein. With the micro-method these studies have been extended to a large number of clovers and the results will be given in a further communication. The genistein content of many samples of the *Dwalganup* strain has now been determined and these results will be available for publication shortly. The figure for *Dwalganup* in Table 2 is a good representative one.

DISCUSSION

The preparation of the 'chloroplast' fraction of clover, although of use in providing a concentrate in readily stored form, is effective in retaining only about 3 % of the active compound. Quantitative estimations of the intake of genistein by sheep, based on the amount present in this fraction, have been most misleading.

The isolation of substantial amounts of genistein from fresh subterranean clover, however, makes it likely that this *iso*flavone is responsible for the breeding problem in sheep maintained on pastures dominated by this plant species. A sheep eating 1 kg. of dry matter per day would have an intake of about 7 g. genistein daily. Genistein, on oral administration in mice has a median effective dose (M.E.D.) of 8·2 mg. (Biggers & Curnow, 1954) so the sheep receives about 850 mouse M.E.D.'s of genistein daily. With injected stilboestrol complete infertility is produced in ewes receiving 0·1 mg. or approx. 4000 mouse M.E.D.'s per day for 6 months (Underwood, Shier & Peterson, 1953). It is not unlikely that a degree of infertility equivalent to that often induced by clover grazing (20–60 %) would result from the injection of 0·02 mg. (approx. 800 mouse M.E.D.'s) of stilboestrol daily, especially if the treatment were carried on for more than one season, as occurs with clover ingestion. Although it is dangerous to make assumptions regarding the activity of genistein in the sheep from the behaviour in mice, it is reasonable to suppose that a somewhat similar response to that with stilboestrol will be given by the daily 850 mouse M.E.D.'s of genistein. It is possible also that genistein is transformed in the rumen of the sheep to a more active oestrogen. There is evidence (Beck & Curnow, unpublished data) that treatment of clover 'chloroplast' with alkalis yields a small amount of a second oestrogen with at least ten times the activity of genistein.

SUMMARY

1. From the 'chloroplast' fraction of subterranean clover (*Dwalganup* strain) 12 mg. genistein per 100 g. dry matter of clover was isolated.

2. From fresh clover 369 mg. genistein per 100 g. dry matter was isolated.

3. A method of estimation of genistein in plant material and a micro-method of detection of genistein in small amounts of plant material have been developed.

4. Four subterranean clovers studied contain genistein, whereas four other species contained no genistein.

5. The *Dwalganup* strain of subterranean clover contained 740 mg./100 g. dry matter, sufficient to account for the oestrogenic activity of the plant and therefore probably responsible for the infertility in sheep grazing on subterranean clover pastures.

I wish to thank the Western Australian Investigational Committee, Professor E. C. Dodds, Dr C. P. Rhoads and the late Dr K. Dobriner for generous assistance in many ways, also Dr A. R. H. Cole for the infrared spectrometry, Dr Lawrence for the use of the ultraviolet spectrophotometer and Mr K. Gillieatt for able technical assistance.

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A Colorimetric Estimation of Sugars Using Benzidine

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Many colorimetric methods using organic reagents are available for the estimation of sugars, a number of which have been utilized in conjunction with chromatography. For example, a method using aniline phthalate has been described (Blass, Macheboeuf & Núnez, 1950; Bartlett, Hough & Jones, 1951). This method, however, consists of several stages, which is a disadvantage. Methods using concentrated mineral acids are to be avoided if possible, in order to simplify practical procedure, and for this reason the method described by Dubois, Gilles, Hamilton, Rebers & Smith (1951), using a phenol–sulphuric acid reagent, was considered unsuitable for routine analysis.

In connexion with work on gums, it was considered necessary to have available a simple colorimetric estimation applicable to all the monosaccharide constituents of complex polysaccharides and their methylated derivatives.

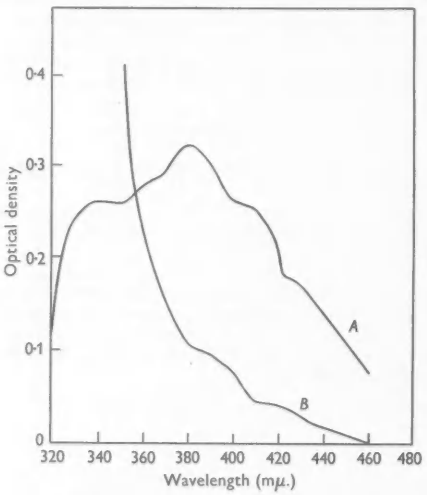

Fig. 1. Absorption spectra for: *A*, reaction of 100 μg. D-galactose with the benzidine reagent; *B*, the blank determination. Curves for aldopentoses, methyl aldopentoses, hexuronic acids, and methylated sugars were similar to *A*.

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Benzidine is used as a chromatographic spray reagent for reducing sugars (Horrocks & Manning, 1949) and for the estimation of pentoses by an indirect method involving furfural (McCance, 1926). An attempt was therefore made to estimate sugars directly using a solution of benzidine in glacial acetic acid. Preliminary experiments showed that aldopentoses, methyl aldopentoses, aldohexoses, hexuronic acids, methylated sugars, and oligosaccharides all gave an orange-yellow coloration when heated with the reagent, and that the colour in each case had a similar maximum absorption band (Fig. 1). Further investigation showed that the relationship between absorption and concentration was linear in all cases studied.

It was observed that the benzidine reagent in glacial acetic acid gradually became pale yellow when exposed to air and light. However, the addition of a small amount of stannous chloride prevented this discoloration, and also increased the sensitivity, enabling the determination of much lower concentrations of sugar. Stannous chloride is also used in this laboratory to stabilize the *p*-anisidine hydrochloride spray reagent (Hough, Jones & Wadman, 1950).

The composition of polysaccharides was determined using the benzidine reagent, after hydrolysis and separation of the monosaccharide constituents on paper chromatograms.

EXPERIMENTAL

Reagent. This consists of a 0·2% (w/v) solution of benzidine (A.R.) in glacial acetic acid (A.R.) containing 0·1% (w/v) $SnCl_2$.

Owing to the slight instability of the reagent, the determination of sugars is not based on a standard curve, but estimations of standard sugar solutions are carried out together with the unknown. The solution of benzidine in glacial acetic acid should be freshly prepared. The addition of stannous chloride produces a cloudy precipitate which is filtered off. The clear, colourless solution is then ready for use.

Method

The estimations are carried out in test tubes (7 × 1 in.) provided with small glass spheres to prevent the entry or loss of water. Sugar solution to be determined (1 ml.), containing not more than 600 μg. of sugar, is added from a pipette into a tube, followed by the benzidine reagent (5 ml.) from a burette. After a thorough mixing, the tubes are heated in a vigorously boiling water bath for a period depending on the class of the sugar (Table 1 and Fig. 2). At

least two standards of suitable concentration are included and also a blank determination, i.e. 1 ml. of water and 5 ml. of benzidine reagent.

After being heated, the tubes are cooled in running water for not less than 3 min. The colour is then measured with a Hilger 'Spekker' photoelectric absorptiometer using a violet filter (Ilford no. 601, wavelength 425 mμ.) and a 0·5 cm. glass cell. The concentration of the unknown can then be calculated from the standards by simple proportion, or, better still, if three or more standards are employed, by plotting absorption against concentration and reading the unknown from the resulting graph. The relation between concentration and absorption was found to be linear over the range 100–600 μg./ml. for the sugars listed in Table 1.

Concentrations as low as 20 μg./ml. can also be determined satisfactorily, provided that standards are included within the range 20–100 μg./ml., and the calculation is based on these standards. The reason for this is that below a concentration of 100 μg./ml. the slope of the concentration/absorption graph changes slightly (Fig. 3). The method is not suitable for concentrations of less than 100 μg./ml. of methylated sugars and disaccharides or less than 50 μg./ml. of hexuronic acids. The determination may be carried out with errors of less than ±3%.

Table 1. *Times of heating sugars with the benzidine reagent*

Sugar	Heating time necessary for maximum colour production (min.)
Arabinose	15
Ribose	
Lyxose	
Xylose	
Glucose	30
Galactose	
Mannose	
Rhamnose	
Fucose	
Glucurone	
Galacturonic acid	
2:3:4-Trimethylxylose	60
2:4:6-Trimethylglucose	
2:3:6-Trimethylglucose	
2:3:4:6-Tetramethylglucose	
Maltose	
Sucrose	

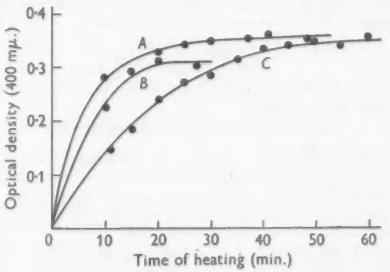

Fig. 2. Variation in optical density (400 mμ.) with time of heating for: *A*, D-glucurone (300 μg.); *B*, D-ribose (250 μg.); *C*, 2:4:6-trimethyl-D-glucose (160 μg.) with benzidine reagent. Methyl aldopentoses, aldohexoses, and galacturonic acid gave curves similar to *A*. All aldopentoses resembled curve *B* and all methylated sugars curve *C*. Measurements were made with a Unicam SP. 350 spectrophotometer using a 1 cm. glass cell.

The absorption spectra of the coloured compounds produced by interaction of the benzidine reagent with sugars were plotted at intervals of 5 mμ. using a Unicam S.P. 500 spectrophotometer, and a 1 cm. glass cell. The maximum absorption with all the sugars tested was in a wavelength band ranging from approximately 340 to 420 mμ. (Fig. 1). Small peaks were evident within the wavelength band, with a small variation depending on the nature of the sugar. The main absorption plateau, however, was of a similar nature in every case. The use of the 'Spekker' with a violet filter to measure the colour produced, instead of a spectrophotometer employing a diffraction grating for the selection of a definite wavelength, was found to be quite satisfactory. The former instrument was easier to operate, especially when dealing with many determinations, and the violet filter allowed absorption to be measured over a wavelength band where the absorption maximum of the blank determination was at a minimum.

Specificity of coloration

The benzidine reagent was observed to give colours with hexose diphosphate, 2-deoxy-D-galactose, dihydroxyacetone, and with ketonic compounds with free or easily accessible carbonyl groups, e.g. acetone, formaldehyde, α-oxoglutaric acid, etc. D-Fructose, however, only gave a colour when in high concentration.

No colour was observed with glycine (in concentration less than 1·0 mg./ml.), tryptophan, tyrosine, asparagine, cystine, hydroxyproline, urea, hexitols, or calcium gluconate. D-Glucosamine hydrochloride gave a faint colour in a concentration of 500 μg./ml.

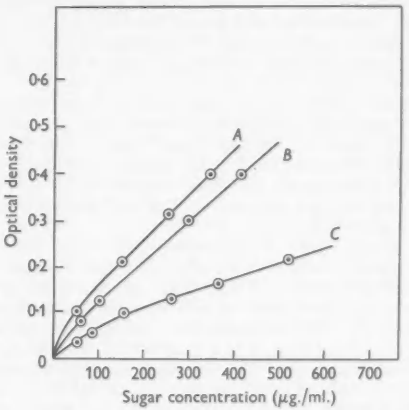

Fig. 3. Concentration/absorption graphs showing change of slope below a concentration of 100 μg./ml. for: *A*, D-mannose; *B*, D-xylose, *C*, D-glucurone. Other sugars show a similar change. The filter used (Ilford 601) transmits light in the range 385–470 mμ., max. transmission 425 mμ.

Table 2. *Analysis of polysaccharides*

Polysaccharide	Ratio of D-glucose or D-galactose to D-mannose: Determined by benzidine method	Ratio of D-glucose or D-galactose to D-mannose: Results of previous workers	Recovery based on ribose recovery (%)
Lucerne seed galactomannan	1:1·35	1:1·25*	101·8
Lilium umbellatum glucomannan	1:1·88	1:1·83†	94·4

* Andrews, Hough & Jones (1952).
† Andrews, Hough & Jones (unpublished results).

Table 3. *Recoveries of sugars after partition on paper*

Sugar	Amount added to paper (μg.)	Recovery (μg.)	Recovery (%)
L-Arabinose	309	309	100
	233	235	101
	155	165	106
D-Xylose	998	930	93
	750	750	100
	499	457	92
	250	229	92
D-Galactose	99	99	100
	398	365	92

Analysis of polysaccharides

Two polysaccharides have been hydrolysed, their constituent sugars separated on paper chromatograms (Flood, Hirst & Jones, 1948) and analysed using the benzidine reagent. The results obtained (Table 2) were comparable with those of other workers.

The sugars were eluted from the chromatograms by shaking the paper strips with suitable volumes of distilled water in tubes fitted with ground-glass stoppers. The sugar solutions were then poured off, centrifuged to remove small particles of cellulose, and samples taken for analysis (cf. Roudier, 1951).

The recovery from the paper using the above technique was examined by applying standard sugar solutions to chromatograms with an 'Agla' micrometer syringe (Burroughs Wellcome and Co., London), developing with ethyl acetate–acetic acid–water solvent (9:2:2, v/v) and eluting the sugar as described. The error was found to be within ±9% (Table 3).

Lucerne seed galactomannan. The polysaccharide (11·38 mg.) was hydrolysed with N-H_2SO_4 in a sealed tube overnight at 100°. D-Ribose (2·925 mg.) was added to the hydrolysate and then the acid neutralized with barium carbonate. After filtration, the solution was evaporated under reduced pressure to a small volume. The three component sugars were then separated chromatographically using *n*-butanol–ethanol–water solvent (40:11:19, v/v) (see Table 2).

Lilium umbellatum *glucomannan.* The glucomannan (20·27 mg.) was hydrolysed in a sealed tube with 98% formic acid at 100° overnight. The acid was removed by evaporation under reduced pressure, and the residue taken up in N-H_2SO_4. D-Ribose (6·318 mg.) was then added and the solution heated at 100° for 30 min. to hydrolyse formyl esters. The acid was then neutralized as before with barium carbonate, and the remainder of the procedure repeated as described for lucerne seed galactomannan (see Table 2).

SUMMARY

1. A new method of determining sugars colorimetrically is described, the reagent consisting of a solution of benzidine in glacial acetic acid. The method is suitable for the determination of aldopentoses, methyl aldopentoses, aldohexoses, hexuronic acids, methylated sugars, and disaccharides. Ketoses and amino sugars cannot be estimated with this reagent.

2. From 20 to 600 μg./ml. of aldopentose, methyl aldopentose, and aldohexose may be determined by the method. Hexuronic acids can be estimated in concentrations ranging from 50 to 600 μg./ml. Methylated sugars and disaccharides cannot be estimated satisfactorily below a concentration of 100 μg./ml. The sugars may be determined with errors less than ± 3 %.

3. The analysis of a glucomannan and galactomannan has been carried out using this procedure.

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Lipid Requirements of the Larva of the Housefly *Musca vicina* (Macq.) Reared under Non-aseptic Conditions

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Up to the present, no nutritional studies of the genus *Musca* have been made, despite the fact that *M. domestica* is extensively used for the laboratory testing of insecticides, and that in recent years, with the development of resistance to insecticides, the importance of physiological studies of the housefly has increased.

In general, the nutritional requirements of insects for lipids are mostly restricted to the unsaponifiable fraction (Scoggin & Tauber, 1950), although the saponifiable fraction (fatty acids) has been shown by Fraenkel & Blewett (1947) and by Pepper & Hastings (1943) to be related to normal growth, wing development, diapause and sterility.

The part played by the sterols in the nutritional requirements of several insects has been studied under non-sterile conditions; blowfly larvae by Hobson (1935) and *Dermestes*, *Tribolium*, *Lasioderma*, *Silvanus*, *Ptinus* and *Ephestia* by Fraenkel & Blewett (1943). As far as is known, bacteria neither synthesize nor require sterols (Deuel, 1951), although some yeasts contain phytosterol.

Our own experiments preliminary to the present work indicated that the bacterial flora of our laboratory-bred housefly failed to make good the nutritional deficiencies suffered by the larvae when reared on a fat-free diet. With this information, an investigation was undertaken into the lipid requirements of *M. vicina*. Some of the results of our work have already been reported briefly in a preliminary note (Bergmann & Levinson, 1954).

MATERIALS AND METHODS

A laboratory-bred strain of *M. vicina* provided the fly eggs and larvae used in the present experiments. The source of the stock bacterial flora and the method of its maintainance have already been described by Silverman & Silverman (1953).

For each test diet, 25 g. of a dry test medium were mixed with 25 ml. of sterile distilled water. The resulting 50 g. of wet medium were divided among seven test tubes (150 × 15 mm.), so that each tube contained approximately 7 g. of medium. Several hundred fly eggs, up to 12 hr. old, were washed in distilled water and added to a suspension of stock bacterial flora in a Petri dish; the suspension contained between 1·5 and 2·5 × 10^8 bacteria/ml., of which 70% consisted of *Escherichia coli* and the remainder mainly of *Sarcinia*, *Lactobacillus* and *Bacillus subtilis*. By means of a pipette, 20–25 eggs in 1 ml. bacterial suspension were transferred to each of the test tubes containing wet medium, which were then incubated at 35°. The internal temperature of the test tube during the period of larval growth was 36°. To ensure that different diets received samples from the same mixture of eggs and bacteria, seven to ten diets were tested at the same time. One test tube out of the seven used for each diet was opened for examination on successive days until the time of pupation. At pupation time (usually the 5th to 7th day) the remaining tubes were opened and the pupae were pooled for weighing. Records were kept of the number and average weight of the larvae recovered daily. The mean weight value for each point on the curve was calculated from the collected average daily weights of larvae reared in replicate experiments. Each mean growth curve in Fig. 1 represents the growth of 1500 larvae. To allow for the differences in the age of the eggs, slight adjustments were made between the growth curves obtained from different series of the same test diet.

Control diet (*no.* 1). This consisted of wheat bran, obtained from the Golan Flour Mills, Tel Aviv. Before use, the bran was covered with pure ether and the ether was allowed to evaporate in a stream of air.

Basic lipid-free diet (*no.* 2). This diet was prepared by extracting 200 g. wheat bran with seven successive 1·5 l. portions of ether and then with one portion of 0·75 l. $CHCl_3$. The extraction time with each portion of solvent was at least 24 hr. The $CHCl_3$ extract (concentrated to 20 ml.) was tested for sterols by the Liebermann-Burchardt reaction (Fieser & Fieser, 1949). In each sample, the extract proved to be sterol-free, thus indicating that the extraction with ether had been complete.

Basic lipid-free diet supplemented with total lipids (*no.* 3). The total lipids were recovered from the extracting solvents mentioned above and added to the basic lipid-free diet no. 2. The added lipids (4% of the total weight) were dissolved in ether, poured on to the dry media, and the ether, which was just sufficient to cover the media, was evaporated, leaving an evenly distributed residue.

Basic lipid-free diet supplemented with yeast extract (*no.* 4). This consisted of diet no. 2 plus 1% dried yeast extract. The yeast preparation, a water-extract of lysed yeast cells, was obtained from the Baltimore Biological Laboratories, U.S.A.; it was tested and found to be free from sterols.

Basic lipid-free diet supplemented with the saponifiable lipids (*no.* 5). This diet consisted of diet no. 2 plus 1% of the saponifiable wheat-bran lipid. The technique of Hilditch (1947) was followed in preparing the saponifiable fraction.

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Care was taken during the saponification and the subsequent distillation of the extracting solvent to prevent oxidation of the fatty acids by keeping the material in an atmosphere of CO_2.

All the other diets were prepared from diets nos. 2 and 4 with supplements.

Diets nos. 1–8 were designed to test the nutritional value of the ether-soluble vitamins, and the saponifiable and unsaponifiable fractions of the lipids contained in the wheat-bran ether extract. Diets nos. 9–14 were prepared in an attempt to investigate the active constituent of the unsaponifiable lipids. The results are summarized in Fig. 1.

RESULTS

It was found that growth curves of replicate batches of larvae reared on the same diets showed variations of ± 10 % at each point on the curve, while pupation times differed by ± 5 %. Larvae reared on different diets and whose resultant growth curves and pupation times differed from one another within these limits are represented by a single curve; e.g. in Fig. 1, curve *B* for diets nos. 3 and 8, curve *C* for diets nos. 9–13, curve *D* for diets nos. 2 and 5, and curve *E* for diets nos. 4 and 7.

Total lipids. A comparison of growth curves *A* and *D* in Fig. 1 shows the reduced growth rate and the lower maximum weight of larvae reared on a lipid-free diet. 3 % of the larvae reared on diet no. 2 (curve *D*), pupated on the 7·5th day, 2 days after 90 % of the larvae reared on the control diet no. 1 (curve *A*) had pupated. When the total lipids were replaced, as in diet no. 3, the resultant growth rate and maximum weight (curve *B*) were greater than those observed in the control larvae.

Ether-soluble vitamins. The effect on larval growth of the ether-soluble B vitamins is demonstrated by the growth curves resulting from diets no. 2 (curve *D*) and no. 4 (curve *E*). The yeast-extract supplemented diet no. 4 supported growth of the larvae at a higher rate than the lipid-free diet no. 2. Although only 10 % of diet no. 4 larvae pupated, pupation took place a full day before those reared on the lipid-free diet. The lower maximum weights of larvae reared on diet no. 4, as compared with those on diet no. 2 may be due to the earlier pupation time.

Saponifiable lipids. Larvae reared on diet no. 5 did not differ from those reared on the lipid-free diet (curve *D*). When larvae were reared on diet no. 7 (the lipid-free diet supplemented with yeast-extract and saponifiable lipids), the resulting growth was the same as that obtained on diet no. 4.

Unsaponifiable lipids. Curve *F* demonstrates the growth of larvae reared on diet no. 6. 50 % of the larvae pupated on the 6th day. In the presence of yeast-extract (diet no. 8) the unsaponifiable fraction

Table 1. *Other diets*

Diet no.	Basic diet no.	Supplements
6	2	Unsaponifiable wheat-bran lipids (0·17 %)*
7	4	Saponifiable wheat-bran lipid (1 %)
8	4	Unsaponifiable wheat-bran lipid (0·17 %)
9	4	Wheat-bran sterol (0·17 %)†
10	4	Sitosterol (0·17 %)
11	4	Cholesterol (0·17 %, m.p. 149°)
12	4	Cholesteryl acetate (0·17 %, m.p. 114°)
13	4	Cholest-4-en-3-one (0·17 %, m.p. 81°)
14	4	Cholestan-3-one (0·17 %, m.p. 129°)

*Recovered from saponification carried out in preparation of diet no. 5.

† Prepared from the unsaponifiable fraction according to the digitonin precipitation method of Windaus (Abderhalden, 1925). The sterols were identified spectrographically (Fieser & Fieser, 1949) as consisting largely of sitosterol. A solution in cold $CHCl_3$ saturated with $SbCl_3$ was yellow, having an absorption max. at 500 mμ. The infrared spectrum was also determined on 12 mg. of sterol in 1 ml. CS_2, using a 1 mm. NaCl cell.

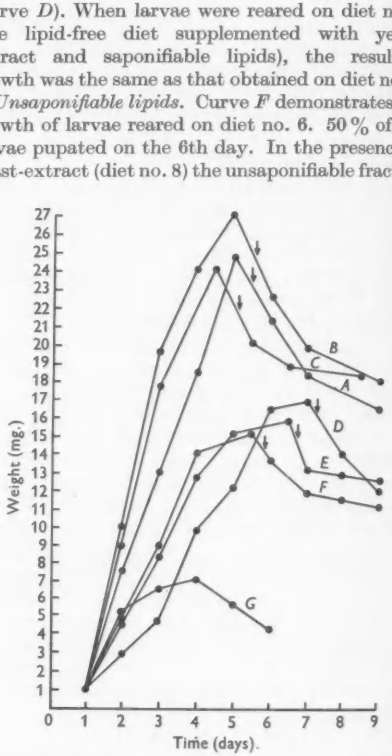

Fig. 1. Mean growth curves and pupation times (↓) of *M. vicina* larvae reared on various diets: *A*, diet no. 1; whole wheat bran. *B*, diets nos. 3 and 8; lipid-free wheat bran re-combined with the total lipid extracted, and lipid-free wheat bran vitamin enriched and supplemented with unsaponifiable fat. *C*, diets nos. 9–13; liped-free wheat bran, vitamin enriched and supplemented with various sterols. *D*, diets nos. 2 and 5; lipid-free wheat bran supplemented with saponifiable fat. *E*, diets nos. 4 and 7; lipid-free wheat bran vitamin enriched and supplemented with saponifiable fat. *F*, diet no. 6; lipid-free wheat bran supplemented with unsaponifiable fat. *G*, diet no. 14; lipid-free wheat bran vitamin enriched and supplemented with cholestan-3-one, and whole wheat bran supplemented with cholestan-3-one.

had a profound effect on larval growth. Diet no. 8, like diet no. 3 (curve *B*), produced larvae which were larger than those obtained on the control diet (curve *A*).

Sterols. When combined with yeast-extract, wheat-bran sterols (diet no. 9) sitosterol (diet no. 10), cholesterol (diet no. 11) cholesteryl acetate (diet no. 12) and cholest-4-en-3-one (diet no. 13) furnished a satisfactory medium for the larvae. Curve *C* is for diet no. 9; very similar curves were obtained with diets 10–13. Pupation took place on the 5th day, the earliest recorded pupation time. Cholestan-3-one, however (diet no. 4, curve *G*), appeared to inhibit growth and pupation, the larvae usually dying between the 6th and 7th day. The inhibiting activity of cholestan-3-one was found to be effective even in the presence of sitosterol.

Effect of temperature. Fig. 2 demonstrates the effect of temperature on the growth and pupation of *M. vicina* larvae. The larvae reared under the constant temperature of 36° grew faster and pupated 2 days before larvae reared in the same media, but subject to changing fermentation temperature which varied from room temperature on the first day to 42° on the 8th day. The growth represented by the variable temperature curve is typical of larval growth recorded from the usual laboratory housefly-breeding techniques which do not control the fermentation temperatures of the medium.

DISCUSSION

The general need of insects for a dietary lipid is confirmed for *Musca vicina*. Since by separating and then recombining the lipid and non-lipid fractions of wheat bran as in diet no. 3, one is able to produce a medium which is more efficient than the whole wheat bran, it appears that the more lipid is available, the more effective is the diet.

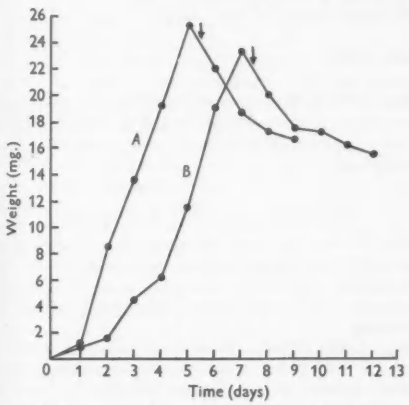

Fig. 2. Mean growth curves and pupation times (↓) recorded from *M. vicina* larvae reared in wheat bran: (*A*) at a constant temperature of 36°; (*B*) at an uncontrolled fermentation temperature varying from 30 to 42°.

It was not possible to prevent, during preparation of the lipid-free diet, extraction of some growth factors not usually considered as lipoidal substances. Certain of the B vitamins required by insects are soluble in fat solvents, e.g. thiamine, riboflavine, pyridoxine, pantothenic acid and *p*-aminobenzoic acid. These were removed to some extent along with the lipids by the extraction process. It appears from growth obtained on diet no. 4, that the role of these growth factors is insignificant when they are added in the absence of lipids.

It becomes clear from the results of diets nos. 5 and 7 that the saponifiable fraction of the wheat-bran lipids are not required by housefly larvae for growth and pupation. On the other hand, the unsaponifiable fraction is very active. The unsaponifiable fraction is unable, however, to act fully in the absence of the B vitamins. The effect of the unsaponifiable fraction could also be produced by its sterol component. The wheat-bran sterol, which is mainly sitosterol, is a relatively non-specific requirement since it, in turn, could be replaced by cholesterol, cholesteryl acetate and cholest-4-en-3-one. The corresponding saturated ketone, cholestan-3-one, appears to act as an antagonist. Fraenkel, Reid & Blewett (1941), van't Hoog (1935) and Hobson (1935), working with *Dermestes*. *Drosophila* and *Lucilia*, have also shown that a wide variety of sterols can satisfy the respective lipid requirements. It is therefore interesting to note that two closely related steroids, such as cholestan-3-one and cholest-4-en-3-one, have such markedly different effects. Whereas cholest-4-en-3-one is able to satisfy the sterol requirement of *Musca vicina*, it has proved to be inhibitory for larvae of *Aedes aegypti* (Golberg & De Meillon, 1947).

The steroids appear to act as both growth and pupation factors for *Musca vicina*. Both Gay (1938) and Golberg & De Meillon (1947), working with *Dermestes* and *Aedes* respectively, have shown that the effect of fat in the larval diet is manifest principally at the time of pupation and metamorphosis, which suggests that the sterol requirements are closely related to the moulting and pupation hormones demonstrated by Wigglesworth (1950) and Fraenkel (1935).

From diets which lacked a source of sterol, a number of flacciform larvae were usually recovered, which were unable to resist infection from pathogenic bacteria. Hobson (1935) also observed this susceptibility to infection, correlating the resistance of blowfly larvae to bacterial infection with the presence of cholesterol.

SUMMARY

1. The lipid requirements of the larvae of the housefly, *Musca vicina* have been studied. The only lipid required is contained in the sterol fraction of the wheat-bran medium.

2. This sterol was identified as sitosterol; however, cholesterol, cholesteryl acetate and cholest-4-en-3-one were found to produce the same biological activity as sitosterol. Cholestan-3-one, on the other hand, does not replace cholesterol; moreover, it is an antagonist of the normal 'active' steroids, inhibiting growth and preventing pupation.

3. The role of the sterol as an agent in larval resistance to bacterial infection, as a pupation factor, and as a promoter of growth is discussed.

4. The experimental rearing method demonstrates that a constant temperature of the medium can accelerate growth and pupation when compared with the usual breeding methods in which fermentation temperatures of the medium are uncontrolled.

Acknowledgement and thanks are made to Professor Ernst D. Bergmann whose aid and counsel made this work possible, and to Miss Judith Byk for technical assistance.

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Studies on the Lipids of *Musca vicina* (Macq.) during Growth and Metamorphosis

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It has been shown in a previous paper (Silverman & Levinson, 1954) that *Musca vicina* requires a sterol as its sole essential 'lipid'; it is apparently unable to synthesize sterols. It was found that larvae could develop to the third stage when reared on a sterol-free diet, but were well below the average normal weight and failed to pupate. In order to determine the relation of sterols and other lipids to the developmental periods of the normal housefly larva, a systematic analysis of lipid at various stages of development was undertaken. It was felt that such an analysis might yield useful information in regard to *M. vicina's* ability to utilize and synthesize lipids. Similar studies by Frew (1929), Evans (1932), Yuill & Craig (1937) and Haub & Hitchcock (1941) on blowfly larvae have been mainly concerned with changes in the saponifiable fraction of larval fat. Evans (1934), however, recorded observations on the unsaponifiable fat of *Tenebrio molitor* during metamorphosis and found that it remained constant throughout.

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MATERIALS AND METHODS

Housefly breeding. The technique for stabilizing the microflora and density of larvae breeding in the medium has been described in full in a previous paper (Silverman & Silverman, 1953). A short summary of the technique is given here.

The housefly larvae were reared in round glass containers, 20 cm. in height and 15 cm. in diameter. The breeding media consisted of the following thoroughly mixed ingredients: 300 g. of wheat bran, 40 g. straw and 300 ml. water. The jars were covered with a double layer of cloth towelling which was held in place by a metal rim. They were sterilized by autoclave and then allowed to cool to room temperature before seeding with eggs. Each jar was seeded with 1000–1250 eggs in 20 ml. water suspension of

the stock bacterial flora ($1{\cdot}5$–$2{\cdot}5 \times 10^8$ bacteria/ml.) in which *Escherichia coli* represented 70% of the total; *Sarcina*, *Lactobacillus* and *Bacillus subtilis* constituted the remaining 30%. Only eggs which were known to have been oviposited within 6 hr. were selected for seeding. The rearing containers were kept at 27° and 60% relative humidity. Between 65 and 75% of the eggs developed to third-stage larvae, so that on the average, 1 g. of rearing media supported 1·5 mature larvae. Whenever practical, fresh material was collected and immediately submitted for analysis. When this was not possible, the material was stored at −24°, until required.

The following stages of the life cycle of the housefly were analysed.

Eggs. These were collected within 6 hr. after being oviposited on clean damp cotton. About 30 g. of eggs were analysed in batches of 5 g.

Third-stage larvae. These were collected at two different time intervals. Early third-stage larvae were collected from the 4th to the 5·5th day and the late third-stage larvae were collected during the period between the 5·5th day and the 7th day. The larvae were allowed to crawl around in Petri dishes for 4–5 hr. so that they might empty their alimentary tracts of all plant material. At least 100 g. of each larval stage were analysed in batches of 5–15 g.

Pupae. These were collected at two different times. Immature pupae, which were white to pink in colour, were collected from the 7·5th to the 8·5th day. Mature pupae, dark brown in colour, were gathered from the 8·5th to the 10th day. All pupae were washed in water to cleanse them of adhering medium. At least 100 g. of each pupal stage were analysed in batches of 5–15 g.

Unfed adults. About 50 g. of unfed adults of both sexes, collected within 2 hr. after emergence, were analysed in 10 g. batches.

Rearing media. Analyses were carried out on 1000 g. samples of rearing media, before and after larval breeding, and on 200 g. of media which had been cultured with only the bacterial flora.

Water content. Moisture content determinations were carried out in three different ways: 5 g. samples were (i) dried in a vacuum desiccator (kept below 25° to minimize oxidation of fats), (ii) oven-dried (80–100°) and (iii) subjected to toluene distillation (Bidwell & Sterling, 1925). The results obtained from the three methods were the same; for purposes of convenience, vacuum desiccation was adopted as the standard drying technique.

Lipid content. The desiccated material was ground with glass powder in an atmosphere of dried CO_2 and transferred to extraction thimbles. The thimbles with their contents were weighed before being introduced into a Soxhlet extraction apparatus whose reflux condenser was attached to an O_2-absorption train.

The extraction solvent was diethyl ether. Before use, it was shaken at intervals over a period of 24 hr. with KOH pellets. The ether was dried over anhydrous $CaCl_2$ and distilled just before use. 250 ml. ether were used for each continuous extraction, which lasted for a period of 48 hr.; these conditions were previously determined as sufficient for completion of the extraction process. After extraction, the thimbles with their contents were weighed and the loss of weight recorded as 'total lipid'. The ethereal extract was stored at 2° until required for further analysis.

Saponification number. The saponification number of the 'total lipid' was determined by the method described by Wizoeff (1927) with a slight modification. The bulk of the solvent was distilled in N_2 by means of a vacuum pump. The remaining solvent containing the total lipids was transferred to weighing bottles and the last traces of ether were removed under a stream of N_2. After obtaining constant weighings, 1–2 g. of the lipid material was dissolved in neutral benzene and saponified for 1 hr. with ethanolic 0·5 N-KOH in alkali-resistant flasks. 0·5 N-HCl and bromothymol blue indicator were used for back titration. Each lipid sample was divided into at least two parts for replicate determinations.

Iodine numbers. The iodine number of the total lipids was determined according to the method of Kaufmann (1940). Depending on the iodine number to be expected, a sample of the ether solution containing between 0·1 and 0·5 g. of lipid was taken for determination. The samples were placed in small tared weighing bottles and dried to constant weight in a stream of N_2. The weighing bottles were placed in 500 ml. glass-stoppered Erlenmeyer flasks and a sufficient amount of halogenating reagent was added so that from 100–150% excess was present. Two to four determinations and blanks were run on each sample.

Unsaponifiable fraction. This was determined by the method recommended by the Fat Analysis Committee of the American Chemical Society (Jacobs, 1951) using the same samples from the determination of the saponification number.

Sterols. Total sterols were determined using Windaus's technique as described by Abderhalden (1925). The factor 0·2563 was used to convert the digitonide into sterol (Windaus, Werder & Gschaider, 1932).

RESULTS

Table 1 summarizes the results obtained from the moisture and lipid analyses of the various stages of the housefly and of its rearing medium. Variations between different batches of the same material were within the limits indicated in the table.

DISCUSSION

Water content. The differences in water content between the various stages were insignificant although a drop was noted during pupation. This apparent loss of water, however, may be the result of the formation of the puparium with its very low moisture content. The moisture content of the newly emerged adult was the same as that of the late third-stage larva indicating (with the limits of experimental error) that a stable water balance is maintained during growth and metamorphosis. Yuill & Craig (1937) recorded a steady loss of water during the growth of *Lucilia sericata* and a sharp drop at the time of pupation. *Musca vicina* did not show such a wide variation.

Lipid content. The lipid content of *M. vicina* rises in 7 days from 2·5 μg. in the egg to 1225 μg. in the late third-stage larva. Within 24 hr. of the onset of pupation, the fat content drops to 880 μg. The 48 hr. old mature pupa contains 714 μg. fat of which, 2 days later, 294 μg. can be recovered from the emergent housefly and 22 μg. from the puparium.

Table 1. *Moisture and lipid content of various stages of the housefly and its rearing medium*

	Average individual live wt. (mg.)	Water (%)	Lipids								
			Total (as % of live wt.)	Average content (μg.)	Saponification number	Iodine number	Unsaponifiable fraction			Sterol	
							As % of total lipids	Average content (μg.)	As % of live wt.	Average content (μg.)
Variations between batches ...	—	(±5)	—	—	(±2)	(±2)	—	—	—	—	—
Egg	0·1	79·0	2·5	2·5	219·1	55·3	—	—	—	—	—
Larva, early third stage	18·0	77·0	4·6	828·0	—	98·0	3·9	32·3	0·033	8·25	
Larva, late third stage	25·0	75·0	4·9	1225·0	216·8	—	—	—	0·036	7·20	
Pupa, immature	20·0	73·2	4·4	880·0	—	61·5	4·5	39·6			
Pupa, mature	17·0	69·2	4·2	714·0	218·5	58·3	4·9	34·9			
Adult, newly emerged	14·0	75·6	2·1	294·0	225·0	63·8	—	—	—	—	—
Puparium	2·0	50·0	1·1	22·0	—	—	—	—	—	—	—
Media before rearing	—	63·6	1·8	—	206·3	126·0	4·1	—	0·106	—	—
Media with bacteria after rearing larvae	—	60·5	0·4	—	209·0	120·0	—	—	—	—	—
Media after culturing only bacterial flora	—	62·6	1·7	—	—	101·0	—	—	—	—	—

It appears that 400 μg. of lipid is used in the first part of the pupation period and about 400 μg. in the latter part. The finding that fat is consumed at the beginning and end of the period of metamorphosis is in agreement with the observations made on the blowfly (Frew, 1929; Evans, 1932; Yuill & Craig, 1937) and on the beetle (Evans, 1934). The studies by Hitchcock & Haub (1941), Patton, Hitchcock & Haub (1941) and Haub & Hitchcock (1941) on *Phormia* have shown that the secondary drop in fat content during pupation is due to an accelerated conversion of fat into carbohydrate.

Saponification number. No appreciable change in the saponification number was observed during larval growth and pupation of the housefly. Fat of the emergent housefly, however, appears to contain a significantly higher proportion of shorter-chain fatty acids. The longer-chain unsaturated fatty acids may have been broken down or consumed during the late pupal period. Yuill & Craig (1937) reported a sudden drop of the saponification number with the advent of pupation in the blowfly.

Iodine number. The fatty acids of the wheat-bran media are relatively unsaturated. During larval growth they are absorbed and laid down in their unsaturated state and with the onset of pupation they are either saturated or broken down into shorter-chain fatty acids. In consideration of the higher saponification number of the emergent housefly, it is most likely that the acids are broken down before being converted into carbohydrate. The relatively low iodine number found in egg fat indicates that the adult housefly generally produces a saturated fatty acid. Yuill & Craig (1937) and Evans (1932) found that *Lucilia* utilizes unsaturated fatty acids in a similar manner.

Unsaponifiable fraction and sterol. Unsaponifiable lipid is present in third-stage larvae in the same proportion as in the rearing medium. The amount of unsaponifiable lipid remains relatively constant throughout the metamorphosis of *M. vicina*. Repeated analyses did not reveal any significant drop in the sterol content of pupal stages as compared with third-stage larvae. Apparently the sterols are not metabolized, probably acting as emulsifying agents to aid in the absorption and metabolism of fatty acids (Kleiner, 1948).

Rearing-media lipid. At a density of 1·5 larvae/g. of rearing medium, *M. vicina* consumed 78 % of the dietary lipid during its growth. Our experience has shown that *M. vicina* can be reared at a density of up to 7 larvae/g. of medium without reduction in larval size. Their ability to extract fat at a relatively low breeding density is therefore considerable. The bacterial flora apparently metabolizes very little fat, though it is capable of saturating the fatty acids to a significant degree. When cultured on wheat bran in the absence of larvae, the bacterial flora reduced

the iodine number from 126 to 101. The larvae are no doubt consuming a higher proportion of saturated fat than is indicated by analysis of the medium alone.

SUMMARY

1. A study of the changes in the moisture and lipid content of the housefly, *Musca vicina*, during growth and metamorphosis is recorded.

2. The apparent drop in water content at the time of pupation is due to the formation of the puparium with its low moisture content rather than an actual loss in water.

3. The lipid content of *M. vicina* rises in 7 days from 2·5 μg. in the egg to 1225 μg. in the late third-stage larva. Within 24 hr. of the onset of pupation, the fat content drops to 880 μg. The 48 hr. old mature pupa contains 714 μg. fat of which, 2 days later, 294 μg. can be recovered from the emergent housefly and 22 μg. from the puparium. It appears that 400 μg. of lipid is used in the first part of the pupation period and about 400 μg. in the latter part.

4. No appreciable change in the saponification number is observed during larval growth and pupation. The fat of the emergent housefly, however, appears to contain a significantly higher proportion of shorter-chain fatty acids.

5. The amount of sterol and unsaponifiable lipid remains relatively constant throughout growth and metamorphosis.

6. During larval growth, the fatty acids of the wheat-bran medium are laid down in their unsaturated state. With the onset of pupation they are saturated or broken down into shorter-chain fatty acids.

7. 78 % of the rearing media lipid is consumed by the larvae during growth.

Acknowledgement and thanks are made to Professor Ernst D. Bergmann, whose aid and advice made this work possible, to Mr A. Kaluszyner for helpful discussion, and to Miss Judith Byk for technical assistance.

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Changes in Chemical Composition During the Development of 'Cholesterol Fatty Livers'

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Many investigations have been made of the effects of feeding cholesterol to rats. Even a brief review of the work from the laboratories of Schoenheimer, Okey, Channon, Sperry, Cook, and others, would be too voluminous for presentation here. In spite of the many publications in this field, relatively few data are available showing the rate at which the different lipid components accumulate in the liver when different amounts of cholesterol are fed.

The data to be presented show that in female rats consuming a cholesterol-rich, hypolipotropic diet, the percentages of total lipids and of cholesteryl esters in the liver reach more or less limiting values within about 3 weeks and then remain relatively constant (throughout the period of observation, 7 weeks). The livers continue to increase abnormally in size at a constant rate and in this experiment were double the normal weight at the termination of the study.

The impression obtained from the usual method of expressing the results, as percentage of fresh liver weight, is that the excessive deposition of lipids has

come to a stop after about 3 weeks and that a condition of equilibrium has been reached. The present results indicate, however, that actually both glycerides and cholesteryl esters increase in absolute amount throughout the experimental period. The concomitant increase in liver weight occurs at such a rate as to produce the impression of a dynamic equilibrium after the rats have been consuming the rations for about 3 weeks. The material accumulating during these first 3 weeks consisted mainly of glycerides. As time went on, the proportion of glycerides in the material being deposited decreased and the percentage of water increased. Deposition of cholesteryl esters was fairly rapid during the first 3 weeks in both males and females. Males continued to accumulate esters at a considerable rate but in females the rate of deposition appeared to decline. There are not sufficient data, however, to establish this as a real sex difference.

EXPERIMENTAL

The care of the animals, preparation of the diets and analytical procedures have been described by Ridout, Lucas, Patterson & Best (1952). The basal diet, which was essentially free from choline, had the following percentage composition: casein (fat-free, vitamin-free) 8, gelatin 6, soya protein ('Alpha protein', Soya Products Division, Glidden Co., Chicago, Ill.) 6, salt mixture 3, celluflour 2, 'sucrose–vitamin mixture' 1, sucrose 62·0, beef fat 10, corn oil 2, cod liver oil concentrate (Ayerst, McKenna and Harrison, Ltd., Montreal) 0·015 and α-tocopheryl acetate 0·010. The following salt mixture (devised by Lucas & Patterson) is one used in this laboratory, made from salts commercially available in finely powdered form and supplying amounts of mineral believed optimum for growth of rats: 1 kg. contains $CaCO_3$, 110 g.; $CaHPO_4$, 325 g.; K_2HPO_4, 275 g.; $MgSO_4,3{\cdot}5H_2O$, 100 g.; NaCl, 150 g.; ferric citrate, 30 g.; 'trace-element mixture', 10 g. The latter contains (g./100 g.): $CaCO_3$, 70; $MnSO_4,4H_2O$, 19·9; $ZnSO_4,7H_2O$, 3·5; $CuSO_4,5H_2O$, 4·0; KI, 0·05; NaF, 0·05; $Al_2(SO_4)_3,K_2SO_4,24H_2O$, 0·4; $CoCl_2,6H_2O$, 0·05; $Na_2SiO_3,9H_2O$, 2·0 and $NaAsO_2$, 0·1. Celluflour is a non-nutritive material to supply bulk, obtainable from Chicago Dietetic Supply House, Chicago, Ill. The 'sucrose–vitamin mixture' consisted of thiamine hydrochloride, 500 mg.; riboflavin, 250 mg.; pyridoxine hydrochloride, 200 mg.; calcium pantothenate, 1·00 g.; nicotinic acid, 1·00 g.; folic acid, 50 mg.; biotin, 30 mg.; 2-methyl-1:4-naphthoquinone, 100 mg.; *p*-aminobenzoic acid, 10 g.; inositol, 50 g. and finely powdered sucrose (100-mesh) to 1000 g.

Female white rats of the Wistar strain (120–150 g. in weight) were kept in individual cages and fed the cholesterol-containing diet *ad lib.* for varying periods of time. In the first experiment a supplement of 0·5% crystalline cholesterol (British Drug Houses Ltd., London) was added to the basal diet at the expense of sucrose. Groups of ten rats chosen at random were killed after 3, 7, 14, 21, 35 and 49 days, respectively; liver lipids were extracted with hot ethanol and analysed as described previously (Best, Lucas, Patterson & Ridout, 1946).

The effect on liver lipids of feeding diets containing different amounts of cholesterol was investigated in four groups of male rats (100–120 g.). Supplements of crystalline cholesterol (0, 0·2, 0·8, and 1·6%, respectively) were added to the basal diet. The animals were fed *ad lib.* for 3 weeks. Ten rats from each group were killed and ten rats each were continued (for a total of 90 days) on the diets containing 0·2 and 0·8% cholesterol, respectively.

RESULTS

The development of the cholesterol fatty liver in female rats is shown graphically in Fig. 1. Deposition of lipids appears to reach an equilibrium after about 3 weeks when the results are expressed as percentage of fresh liver (Fig. 1*a*). Actually, there is a continued deposition of glycerides and cholesteryl esters as shown in Fig. 1*b*. The average

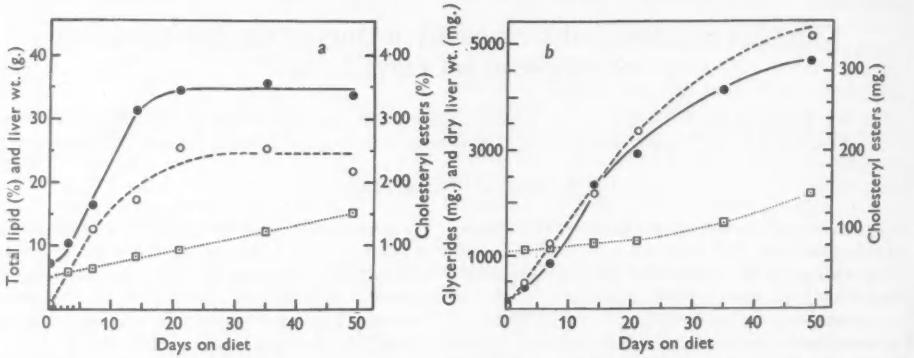

Fig. 1. Rate of accumulation of lipids in livers of rats. Groups of ten rats fed an hypolipotropic diet containing 0·5% cholesterol for periods shown. Data in Fig. 1*a* as % of fresh liver, in Fig. 1*b* as mg. per liver. In Fig. 1*a* left-hand scale gives values for % total lipids (●) and fresh weight of liver (⊡) in g.; right-hand scale % cholesteryl esters (○). In Fig. 1*b* left-hand scale gives values for mg. glycerides (●) and mg. dry, fat-free liver (⊡); right-hand scale gives mg. cholesteryl esters (○).

Table 1. *Changes in composition during the development of 'cholesterol fatty livers'*

Groups of ten female rats (120–140 g.); average food consumption 9–10 g./day; dietary cholesterol 0·5%.

	Days on ration				Material deposited in periods shown					
	0	21	35	49	0–21		21–35		35–49	
Component	Mean weight in g. ± S.E.				(g.)	(%)	(g.)	(%)	(g.)	(%)
Fresh liver	5·57±0·17	9·27±0·91	12·27±1·61	15·93±0·77	3·70	—	3·00	—	3·66	—
Dry, fat-free tissue	1·12±0·02	1·31±0·05	1·47±0·10	2·20±0·12	0·19	5·1	0·16	5·3	0·73	20·0
Total lipids	0·38±0·008	3·40±0·59	4·74±0·87	5·33±0·37	3·02	—	1·34	—	0·59	—
Water (by difference)	4·07±0·14	4·56±0·30	6·06±0·76	8·40±0·61	0·49	13·2	1·50	50·0	2·34	64·0
Glycerides (by difference)	0·17±0·007	2·94±0·58	4·15±0·81	4·68±0·34	2·77	74·9	1·21	40·3	0·53	14·5
Cholesterol plus ester*	0·018±0·0004	0·244±0·028	0·345±0·054	0·376±0·030	0·226	6·1	0·101	3·4	0·031	0·8
Phospholipids	0·191±0·003	0·213±0·010	0·249±0·018	0·275±0·017	0·022	0·6	0·036	1·2	0·026	0·7
						99·9		100·2		100·0

* Cholesteryl esters were calculated as oleate by multiplying bound cholesterol by 1·68.

Table 2. *Composition of material found in livers after feeding cholesterol-containing diets for the periods shown*

Groups of ten male rats (100–120 g.); average food consumption 9–10 g./day.

		Cholesterol in diet (%)					
		0	0·2		0·8		1·6
	At start	21 days	21 days	90 days	21 days	90 days	21 days
Component	Mean weights in g. ± S.E.						
Fresh liver	5·15±0·27	9·19±0·57	9·91±0·59	16·28±1·25	10·68±0·92	21·69±0·82	11·11±0·79
Dry, fat-free tissue	1·08±0·05	1·58±0·10	1·47±0·17	2·17±0·13	1·70±0·10	2·60±0·07	1·80±0·08
Total lipids	0·30±0·014	2·20±0·26	3·01±0·21	5·87±0·54	3·32±0·57	7·94±0·47	3·54±0·43
Water (by difference)	3·77±0·21	5·41±0·29	5·43±0·42	8·24±0·68	5·66±0·30	11·15±0·45	5·77±0·30
Glycerides (by difference)	0·12±0·009	1·98±0·25	2·71±0·20	5·23±0·49	2·91±0·53	6·23±0·42	3·01±0·39
Cholesterol plus ester	0·013±0·0007	0·047±0·006	0·104±0·010	0·359±0·060	0·229±0·027	1·344±0·093	0·338±0·037
Phospholipids	0·169±0·006	0·176±0·006	0·197±0·008	0·281±0·018	0·180±0·014	0·369±0·011	0·188±0·012

weight of the fatty livers at the end of the experiment was 15·9 g. which is just about double that from a normal rat of the same weight. The liver weight of a normal rat on a stock diet may be calculated with reasonable accuracy, being about 4–5 % of body weight. After 49 days on the basal diet the average weight of the rats was 185 g., thus the expected weight of the liver would be about 8 g.

It may readily be shown that the abnormal increase in size of the liver was due mainly to deposition of glycerides and water. At the beginning, the average weight of the rats was 130 g. with liver weight of 5·57 g. Normal female rats of our colony have average total liver lipids of about 6–7 % and an average dry, fat-free liver residue of 20–22 %. The absolute weights of these fractions at the beginning of the experiment were 0·38 and 1·12 g., respectively. By difference, the amount of water in these livers would be 4·07 g. and by calculation the glyceride portion of the lipid was 0·17 g. Values found by actual determination on the livers of rats fed the diet containing 0·5 % cholesterol for 21, 35 and 49 days are included with these data in Table 1. The absolute weights of free cholesterol in the livers did increase slightly but these increments are so small in comparison with the much larger changes in cholesteryl esters that the two have been combined in the table.

During the first 3 weeks most of the increase in weight of the liver was due to deposition of glycerides (74·9 %); the next largest increase was in water (13·2 %). These two components accounted for about 90 % of the materials deposited during the first 21 days, which is the period adopted in most laboratories for studying fatty livers. In the period following the establishment of the apparent equilibrium (Fig. 1*a*) the material being deposited is of a rather different composition although glycerides and water still constitute 80 to 90 % of the increment in weight. The ratio in which they are laid down, however, is greatly altered; about twice as much water as fat is deposited during the interval 21 to 49 days. The ratio in which glycerides, water and other components accompany the deposition of cholesteryl esters after 3 weeks are such as to leave the percentage composition of the liver relatively constant. The material deposited during the period 21–49 days contained about 30 % of total lipids which is essentially the percentage of total lipids in the liver at 21 days. Thus the constancy of the total lipids as percentage of wet weight is explained.

The amount of dry, fat-free tissue increased progressively with the growth of the rat (Table 1). Several interesting trends may be seen in these data. The rate at which total lipids increased fell off and the percentage of water in the material deposited increased. The sum of water plus glycerides remained relatively constant (90 and 79 %) during the periods 21–35 and 35–49 days. The percentage of cholesterol plus cholesteryl esters in the material deposited diminished, as did the absolute amount.

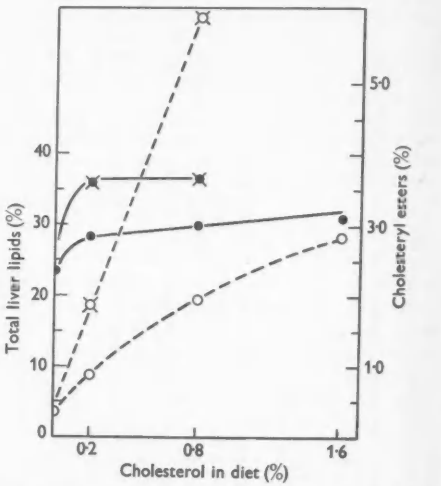

Fig. 2. Influence of dietary cholesterol on liver lipids. Left-hand scale shows total lipids as % fresh liver after rats had consumed rations for 3 weeks (●) and 3 months (✖); right-hand scale gives cholesteryl esters as % fresh liver after 3 weeks (○) and 3 months (□).

Table 3. *Percentage composition of material deposited in livers of male rats under dietary conditions shown*

For experimental details see Table 2 and text.

	Cholesterol content of diet (%)					
	0	0·2		0·8		1·6
Component	0–21 days	0–21 days	21–90 days	0–21 days	21–90 days	0–21 days
Dry, fat-free tissue	12·4	8·2	11·0	11·2	8·2	12·1
Water	40·6	34·9	44·1	34·2	49·9	33·6
Glycerides	46·0	54·4	39·6	50·5	30·2	48·5
Cholesterol plus ester	0·8	1·9	4·0	3·9	10·1	5·5
Phospholipids	0·2	0·6	1·3	0·2	1·7	0·3
Total	100·0	100·0	100·0	100·0	100·1	100·0

Phospholipid was the only fraction which appeared to be relatively constant in its rate of deposition.

The effect of increasing the concentration of cholesterol in the diet of male rats may be seen in Fig. 2, where data for total liver lipids and for cholesteryl esters are presented as percentage of fresh liver weight. More complete data about the composition of these livers are available in Table 2. The increases which occurred in each component were calculated (as in Table 1) and the percentage composition of the material deposited in the livers under each of the dietary regimens was calculated. These data are shown in Table 3. Again it may be seen that regardless of the amount of cholesterol in the diet, the material deposited in the liver consists principally of glycerides and of water. As might be anticipated, the deposition of cholesteryl esters increases with increasing amounts of dietary cholesterol. In these male animals the cholesteryl esters continued to rise throughout the period of observation. At the start of the experiment, the absolute amount of free cholesterol was about 10 mg. and that of cholesteryl esters about 3 mg., making a total of 13 mg. (cf. Table 2). In the case of rats consuming the diet containing 0·8% cholesterol, the absolute amounts of free cholesterol at 21 and 90 days were 20 and 66 mg., respectively, and the corresponding values for cholesteryl esters were 209 and 1278 mg. Since the liver gained 5·53 g. in weight in the first 21 days and cholesterol plus esters account for 216 mg. of this, they represent 3·9% of the material deposited in this interval (cf. Table 3). When rats were maintained on the same diet for a total of 90 days the cholesterol plus ester portion increased to 10·1% of the material deposited during the period of 21–90 days. The female rats used in the first experiment did not exhibit the same increase. Actually, in female rats, the rate at which cholesteryl esters were accumulating decreased with time (after 21 days).

SUMMARY

1. Analyses have been made of the lipids accumulating in the livers of rats fed a hypolipotropic diet containing different amounts of crystalline cholesterol (0, 0·2, 0·5, 0·8 and 1·6%, respectively) and the rates at which the different components accumulate in the liver have been determined.

2. The material deposited during the first 3 weeks consisted mainly of glycerides and of water, these two accounting for between 80 and 90% of the increase in weight of the liver. When the cholesterol-containing diet was fed for longer periods, the nature of the material being deposited changed: the amount of glyceride decreased and that of water increased, but the sum of these two continued to account for about 80% of the gain in liver weight.

3. Although the percentage of total lipids in the livers reached a more or less limiting value after feeding the diets for about 3 weeks, the absolute amount of glycerides and of cholesteryl esters continued to increase throughout the period of observation.

4. In the livers of rats consuming diets containing cholesterol, cholesteryl esters increased about 200-fold in absolute amount, but since the normal concentration is low, the cholesteryl esters never accounted for more than a small percentage of the total lipid material deposited.

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Preventive and Curative Studies on the 'Cholesterol Fatty Liver' of Rats

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Some preventive and curative studies of the so-called 'cholesterol fatty liver' were made almost twenty years ago. This early work has been reviewed by Best & Ridout (1939) and by McHenry & Patterson (1944). Increased knowledge of dietary requirements indicated the desirability of re-investigating this type of fatty liver in rats fed more adequate diets. The preventive effect of choline chloride and of inositol in improved rations containing graded doses of cholesterol was reported recently from this laboratory (Ridout, Lucas, Patterson & Best, 1952). These experiments revealed two points upon which further data seemed desirable: (1) the effect of a change in the nature of the dietary protein, and (2) the effect of somewhat larger doses of choline chloride. Preventive and

curative experiments under these new conditions have now been completed and the curative effects of betaine, of vitamin B_{12} and of inositol have been determined.

The protein portion of the basal diet used in the recent study (Ridout *et al.* 1952) consisted of casein (8 %) and gelatin (12 %). Although gelatin is devoid of tryptophan and almost lacking in tyrosine and cystine, the amount of casein used supplied nearly enough tryptophan for young adult rats. Gelatin contains large amounts of glycine and of proline, which some authors have claimed to be deleterious when present in excess. Half of the gelatin was therefore replaced by soybean protein in the present study. This mixture has the same methionine content as the other diet but provides a better assortment of amino acids by increasing tryptophan, threonine, phenylalanine and histidine. Biotin, folic acid, *p*-aminobenzoic acid and 2-methyl-1:4-naphthoquinone were added to the vitamins supplied previously and a new salt mixture was also adopted. These changes did not affect appreciably the general conclusions. When considerable amounts of cholesterol were present in the diet, even high concentrations of choline chloride in the ration failed to prevent a considerable accumulation of cholesteryl esters in the liver.

The main purpose of the present communication is to describe some curative experiments with lipotropic substances in rats with 'cholesterol fatty livers'. These were produced by feeding (for a preliminary period of 3 weeks) the basal diet to which 0·5 % cholesterol had been added. Cholesterol was then withdrawn from all diets. Some rats were continued on the basal ration; other groups were given different lipotropic substances for various periods of time. In the livers of rats maintained on the cholesterol-free basal diet, there was a gradual fall in cholesteryl esters but glycerides remained high throughout the experimental period of 10 weeks. At the dosage level used, supplements of choline and of betaine were about equally effective in producing a decrease in glycerides. The removal of cholesteryl esters from fatty livers was accelerated by dietary choline or betaine but was still a slow process. Vitamin B_{12} exerted a considerable lipotropic effect upon the glyceride fraction, but the reduction of cholesteryl esters was less definite. Inositol had no significant effect on either fraction.

EXPERIMENTAL

The care of the animals and analytical procedures have been described in previous publications (Ridout *et al.* 1952; Ridout, Lucas, Patterson & Best, 1954); the composition of the basal diet is given in the latter. Inositol was omitted from the 'sucrose–vitamin powder' there described.

Preventive experiment. Male white rats (250) of the Wistar strain (100–120 g. in wt.) were divided among twenty-five comparable groups. The animals, in individual cages, were fed the test diets *ad lib.* for 3 weeks.

Several groups of diets were prepared in which different amounts of crystalline cholesterol (0, 0·2, 0·8 and 1·6 %) were added to the basal ration. Choline chloride was added to certain of these diets in amounts corresponding to 0·04, 0·08, 0·16, 0·32, 0·64 and 1·28 % of choline (factor, choline chloride: choline = 1·15). Supplementary betaine hydrochloride (0·211 and 0·422 %, amounts molecularly equivalent to 0·16 and 0·32 % choline, respectively) was added to two diets containing 0·2 % cholesterol. To another diet containing the same amount of cholesterol, 0·32 % of inositol was added. Four groups were fed the test diets for 3 months; two of these groups were given the ration containing 0·2 % cholesterol with and without 0·64 % choline, respectively; the other groups were fed the ration containing 0·8 % cholesterol with and without 1·28 % choline, respectively.

Curative experiment. Male white rats (140) of the Wistar strain (125–150 g. in wt.) were fed for a preliminary period of 3 weeks, the basal diet to which 0·5 % crystalline cholesterol had been added. At the end of this time the rats were distributed at random among fourteen groups. One group was killed and the liver lipids were analysed to determine the degree of fatty deposition and the amount of cholesteryl esters present. Cholesterol was then withdrawn from the diet of the remaining groups. Four groups were continued on the basal ration for periods of 14, 28, 42 and 70 days, respectively; another four groups were fed for corresponding periods the basal diet to which 0·368 % of choline chloride had been added. An equimolecular amount of betaine hydrochloride (0·422 %) was added to the diet offered to two other groups for 14 and 42 days, respectively. The three remaining groups were fed, for 42 days, diets containing 0·32 % inositol, choline (0·32 %) plus inositol (0·32 %), and 10 μg. of vitamin B_{12}/100 g., respectively.

RESULTS

A comparative study of the effectiveness of the different lipotropic agents was made in rats given the diet containing 0·2 % cholesterol (Fig. 1). Choline and betaine were almost equally effective in preventing the accumulation of total lipids (glycerides), while inositol had no effect. Choline at lower dosages was less effective in preventing the deposition of abnormal amounts of cholesteryl esters; e.g. with 0·08 % of dietary choline the esters accumulated to 5 times the normal concentration, although total lipids were maintained at essentially normal values. A larger dose of choline (0·64 %) did maintain the esters within the normal range at this intake of cholesterol (see Fig. 2). Under these dietary conditions supplementary betaine at moderate dosage (0·32 %) appeared to be as effective as choline, but at lower dosage (0·16 %) it seemed less effective in preventing the accumulation of cholesteryl esters. A proper comparison of their relative lipotropic activity can only be made by constructing more complete dose/response curves. Data are available which indicate that under some conditions betaine is much less effective than choline (Best, Lucas, Ridout & Patterson, 1950).

The main purpose of this preventive experiment was to study the effects of larger doses of choline on the glycerides and cholesteryl esters in the livers of rats ingesting cholesterol-rich diets. Choline is markedly effective in preventing the accumulation of glycerides at all intakes of cholesterol (Fig. 2*a*). In contrast, large doses of choline are unable to prevent a considerable accumulation of cholesteryl esters in the liver when the diet contains excessive amounts of cholesterol (0·8 or 1·6 %). This does not mean that choline has no lipotropic effect on the esters: rats consuming the diet containing 1·6 % cholesterol had liver cholesteryl esters of 17·4 % (on a dry, fat-free basis); these are reduced to 9·3 % by choline. Even prolonged consumption of choline-rich diets does not prevent the deposition of excessive amounts of cholesteryl esters in the liver. For example, when rats were fed a diet containing 0·8 % cholesterol for 3 months, the esters reached 49 % dry, fat-free liver (5·9 % of fresh liver); with 1·28 % choline present in the ration there was a great reduction, although the ester content was still high, viz. 11 % (2·6 % fresh liver). Normal values are about 0·5 % dry, fat-free liver (about 0·1 % fresh liver weight).

Curative experiment. The nature of the lipotropic supplements and their effectiveness in curing the 'cholesterol fatty liver' are presented in Table 1. The lipid components are given as absolute weights (mg. per liver), together with the average fresh liver weights and the average dry, fat-free weights of the liver for each group so that the percentage of each component may be calculated on either basis. The effect on total liver lipids of removing cholesterol from the diet is shown in Fig. 3*a* as percentage of fresh liver weight, and the effect on cholesteryl esters (expressed as percentage dry, fat-free tissue) is shown in Fig. 3*b*. The relative effectiveness of the several lipotropic agents on total lipids and cholesteryl esters may be seen in these figures.

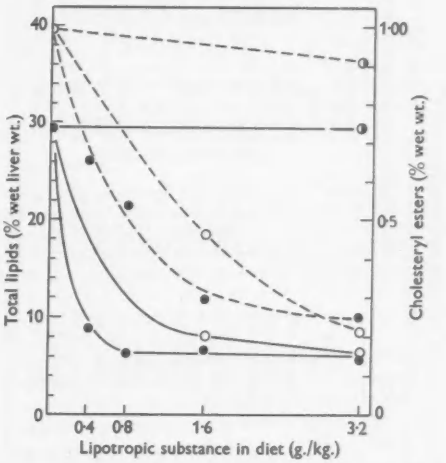

Fig. 1. Comparison of activity of lipotropic agents in diet containing 0·2 % cholesterol. Groups of ten rats fed rations for 3 weeks. Total lipids (—), cholesteryl esters (– – –), choline (●), betaine (○), inositol (◑).

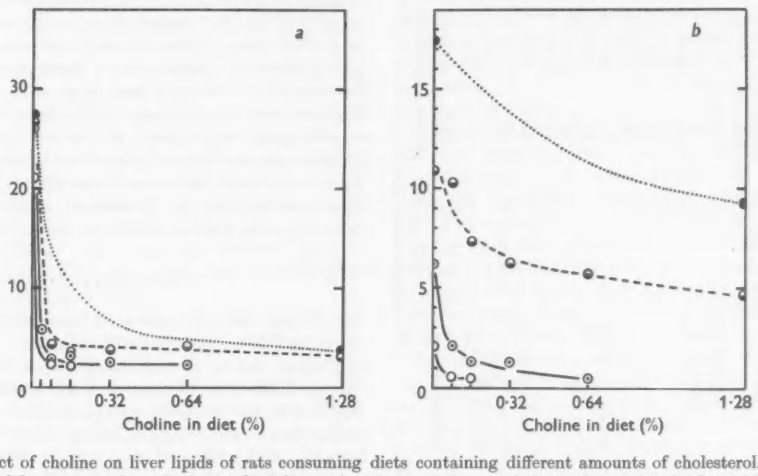

Fig. 2. Effect of choline on liver lipids of rats consuming diets containing different amounts of cholesterol. Curves in left-hand figure show glycerides as % fresh liver when diet contains no cholesterol (○), 0·2 % (⊙), 0·8 % (◒) and 1·6 % (●) cholesterol. Curves in right-hand figure show cholesteryl esters as % dry, fat-free liver under corresponding conditions.

Table 1. *Curative effects of lipotropic supplements in rats with 'cholesterol fatty livers'*

Basal diet supplemented with 0·5% cholesterol fed for a preliminary period of 3 weeks to groups of ten male rats 125–150 g. Average daily intake of cholesterol-free diet during curative period, 10–11 g.

Group no.	Supplements	Period on curative diet (days)	Mean liver weights		Composition of liver lipids (mg./liver)			
			Wet (g.)	Fat-free dry (g.)	Total lipids	Cholesterol		Glyceride
						Free	Total	Ester†	
0	None (basal)	0	10·7 ± 0·7	1·47 ± 0·07	3872 ± 362	22 ± 1·2	203 ± 25	305 ± 41	3303 ± 336
1	—	14	15·1 ± 0·8	1·74 ± 0·08	6261 ± 429	31 ± 2·0	239 ± 23	351 ± 35	5576 ± 388
2	—	28	15·1 ± 0·7	1·87 ± 0·06	6026 ± 436	30 ± 1·2	203 ± 11	292 ± 19	5373 ± 431
3	—	42	18·1 ± 1·0	2·19 ± 0·09	7449 ± 547	35 ± 2·1	182 ± 19	248 ± 29	6817 ± 513
4	—	70	16·2 ± 0·7	2·03 ± 0·06	6384 ± 531	33 ± 1·3	153 ± 9	203 ± 14	5814 ± 520
5	Choline (0·32%)*	14	10·1 ± 0·7	1·64 ± 0·08	2695 ± 295	23 ± 1·4	192 ± 20	284 ± 32	2109 ± 255
6	—	28	8·6 ± 0·4	1·61 ± 0·08	1403 ± 149	19 ± 1·0	110 ± 12	154 ± 19	960 ± 129
7	—	42	7·8 ± 0·4	1·56 ± 0·09	832 ± 99	17 ± 0·8	66 ± 9	83 ± 16	470 ± 86
8	—	70	6·3 ± 0·3	1·36 ± 0·06	452 ± 41	14 ± 0·7	21 ± 2	13 ± 3	192 ± 31
9	Inositol (0·32%)	42	15·5 ± 0·7	1·97 ± 0·05	5693 ± 441	29 ± 1·4	152 ± 12	207 ± 20	5116 ± 421
10	Choline (0·32%)* + inositol (0·32%)	42	7·9 ± 0·5	1·62 ± 0·10	579 ± 85	16 ± 1·2	64 ± 7	80 ± 10	210 ± 55
11	Betaine (0·32%)*	14	11·1 ± 0·5	1·72 ± 0·08	3132 ± 238	27 ± 1·7	189 ± 24	272 ± 39	2528 ± 195
12	—	42	10·1 ± 0·7	1·89 ± 0·11	1096 ± 118	19 ± 0·9	72 ± 10	89 ± 16	672 ± 99
13	Vitamin B_{12} (10 μg.)	42	11·3 ± 0·3	2·03 ± 0·04	2207 ± 140	26 ± 0·9	136 ± 12	186 ± 20	1619 ± 134

* Choline was supplied as chloride (0·368%) and betaine as a molecularly equivalent amount of hydrochloride (0·422%).

† Cholesteryl esters were calculated as oleate by multiplying bound cholesterol by 1·68.

The curves in Fig. 3, however, do not give all the information which is necessary to evaluate properly the relative lipotropic effectiveness of choline, betaine, inositol and vitamin B_{12}. As is shown in Table 1, the livers of the rats fed the basal diet continue to increase in size for 6 weeks after cholesterol is removed from the ration; although the amount of cholesteryl esters decreases, the glycerides (and total lipids) continue to increase. The fact that total lipids and liver weight are increasing in the same proportion gives the erroneous impression (Fig. 3*a*) that in rats on the basal diet no change in total lipids occurs after about 2 weeks.

Although it may seem in Fig. 3 that inositol has failed to exert any significant curative action on either total lipids or cholesteryl esters, the livers are slightly smaller and the absolute weights of these components are reduced proportionately. Vitamin B_{12} had a considerable lipotropic effect: the size of the livers was greatly reduced as well as the percentage of total lipids. The action of vitamin B_{12} was mainly on the glycerides (reduced at 42 days from 6817 mg. per liver in the basal group to 1619 mg.). Some removal of cholesteryl esters from the liver was noted, but the effect of vitamin B_{12} on this fraction was less pronounced. Supplements of choline, of betaine, and of choline plus inositol were about equally effective in hastening the removal of total lipids. Over 6000 mg. of glycerides and about 160 mg. of cholesteryl esters had been removed by each of these supplements at 42 days. Restoration of normal composition is a very slow process, however, for even after a period of 6 weeks on these curative diets, the percentage of glycerides still present in the livers is about twice normal (5·9% fresh liver weight compared with the normal range of 2–4%) and cholesteryl esters are nearly 8 times normal (0·79% compared with the normal range of 0·07–0·14%). The only rats that were kept on a curative diet for as long as 70 days were those receiving the supplement of choline (group 8); in these the percentage of glycerides in the liver was back to normal. However, even after 10 weeks on this curative ration, cholesteryl esters were still slightly above normal (0·20% of fresh liver weight).

DISCUSSION

Lipotropic findings obtained in animals suffering from multiple dietary deficiencies are difficult to interpret. As we have pointed out elsewhere (Best, Lucas, Ridout & Patterson, 1950) the ideal ration for the study of lipotropic phenomena should be complete in every respect, except for the lipotropic agents, which should be absent. Methionine is an essential amino acid and cannot be omitted from the diet, but the lowest concentration of methionine compatible with the general well-being of the rats

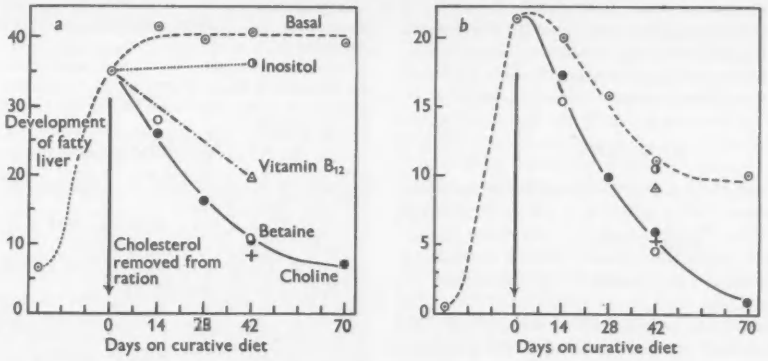

Fig. 3. Development and cure of 'cholesterol fatty liver'. Fatty liver produced by feeding diet containing 0·5% cholesterol for 3 weeks. Groups of ten rats fed curative diets (cholesterol-free) for periods shown. Curves in left-hand figure depict total lipids as % fresh liver in rats continued on hypolipotropic basal diet (⊙), and in rats on other diets containing supplementary inositol 0·32% (◑), vitamin B_{12} 10 μg./100 g. (△), betaine 0·32% (○), choline 0·32% (●) and choline 0·32% plus inositol 0·32% (+). Curves on the right-hand side give corresponding data for cholesteryl esters expressed as % dry, fat-free liver.

has been sought, and all the known essential dietary factors for rats have been included in the rations adopted for these studies. The improved supply of essential amino acids, minerals and vitamins in this more complete ration permitted moderate gains in weight (1–1·5 g./day) where the former diet barely maintained weight. No greater improvement in growth was expected as methionine, which totalled 360 mg./100 g. in both rations, appeared to be one of the limiting amino acids in both diets. The animals on the improved ration seemed healthier, appearing well groomed, bright-eyed and lively.

Even large intakes of choline are unable in preventive studies to maintain liver cholesteryl esters in the normal range when the cholesterol intake is excessive. While this may appear to support the contention that the presence of cholesterol in a diet is deleterious and to be avoided, it is a matter of clinical experience that at reasonable intakes of cholesterol the amounts of lipotropic agents (choline, betaine, methionine and vitamin B_{12}) present in usual mixed rations are adequate to maintain the liver lipids within the normal range (see also Ridout *et al.* 1952).

The impression obtained in these present experiments, that betaine is just as effective as choline in both preventive and curative studies, is probably an artifact due to the lack of data in a suitable dosage range. Under other conditions where data were available to construct a complete dose/response curve, betaine was distinctly less active than choline when the comparison was made, as it should be, on the steep part of the response curves (Best *et al.* 1950).

The first attempt to use choline in the cure of a cholesterol fatty liver was made by Channon & Wilkinson (1934). In the 12-day test period adopted, no beneficial effect of choline was noted. By prolonging the curative period to 41 days, Best & Ridout (1935, 1936) established that choline does accelerate the removal of cholesteryl esters from the liver. They showed that the primary effect of choline is on the glycerides and that the removal of cholesteryl esters is a much slower process. The present paper contains more complete data on the rate of removal of the lipid components under the influence of choline and supplies new information on the curative effects of betaine, of vitamin B_{12} and of inositol. The findings are clear-cut and require no further comment.

The role of vitamin B_{12} in lipotropic phenomena is still not clearly defined. The protective effect of vitamin B_{12} on the renal lesions in young rats described by Schaefer, Salmon & Strength (1949) (see also Strength, Shaefer & Salmon, 1951) has been seen in our laboratory, but in other rats on similar diets with a lower lipotropic background no beneficial effect whatever from vitamin B_{12} was observed (Best, Lucas, Patterson & Ridout, 1953; cf. Stekol & Weiss, 1950). Similarly, in older rats, others have demonstrated that by altering the dietary background one may either obtain or fail to elicit a lipotropic effect with vitamin B_{12} (Drill & McCormick, 1949; McCormick & Drill, 1950; György & Rose, 1950; György, 1951; Bennett, Joralemon & Halpern, 1951). Until more is known about the relationship of vitamin B_{12} to the biosynthesis of labile methyl groups and to the *in*

vivo formation of choline, it is unwise to advance reasons for the inconsistent findings. In the present curative study, the liptropic effect of vitamin B_{12} was very distinct, at least on the glyceride fraction; the effect on cholesteryl esters was less definite.

SUMMARY

1. Crystalline cholesterol was added at different concentrations (0·2, 0·4, 0·8 and 1·6 %) to a hypolipotropic diet. The livers of rats fed these rations and of other rats fed similar rations containing various lipotropic supplements were analysed for lipid content.

2. At moderate intakes of cholesterol (0·2 %) sufficient dietary choline or betaine were able to prevent the excessive deposition of both glycerides and cholesteryl esters.

3. In rats consuming diets rich in cholesterol, even considerable concentrations of choline (1·28 %) in the diet failed to prevent an excessive accumulation of cholesteryl esters in the livers.

4. In a curative experiment, betaine hydrochloride at a concentration of 0·42 % in the diet was about as active as an equimolecular amount of choline in hastening the removal of glycerides and cholesteryl esters from 'cholesterol fatty livers'. The limited data suggest that had the effect of lower dosages been compared, betaine would have been found to be less effective than choline.

5. Vitamin B_{12} exerted a considerable curative lipotropic action on the glycerides but much less on the cholesteryl ester fraction of liver lipids.

6. Inositol was without any appreciable lipotropic effect on either fraction in either type of study.

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Effects of Lipotropic Substances on the Cholesterol Content of the Serum of Rats

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Some investigators believe that there is a causal relationship between atherosclerosis and the level of serum cholesterol. The well-known effect of lipotropic agents upon lipid deposits in the liver has suggested to many workers that these substances might be useful in treating or preventing the lesions in the blood vessels. The literature concerning the effects of lipotropic substances on serum cholesterol is very inconsistent. This prompted us to determine free and total cholesterol in the sera of certain rats used in the lipotropic study reported in the preceding paper (Ridout, Lucas, Patterson & Best, 1954).

Some of the discrepancies in the literature may be related to our observation that under certain conditions, ingestion of diets rich in cholesterol leads to a marked postprandial elevation in the bound serum cholesterol when the food contains choline. Our findings indicate that the same substances that prevent or cure fatty livers are also concerned in the

control of serum cholesterol but they do not provide any support for the claims that lipotropic substances reduce the concentration of cholesteryl esters in the serum.

EXPERIMENTAL

Groups of ten or more rats were fed purified diets (containing casein 8%, gelatin 6% and soybean protein 6%) for periods of from 1 day to 10 weeks, as described in the preceding paper (Ridout *et al.* 1954). Supplements of choline were added as chloride in amounts 1·15 times the amounts mentioned in the text and of betaine as hydrochloride in amounts molecularly equivalent to the choline. At intervals from 2 to 18 hr. after removal of the food containers from the cages, the animals were anaesthetized by intraperitoneal injection of sodium amytal and blood was collected from the carotid artery in 15 ml. conical centrifuge tubes. The blood stood at room temperature for several hours and then remained in a refrigerator overnight. The tubes were centrifuged; as much serum as possible was removed with a pipette and if necessary the serum was again centrifuged. Serum from haemolysed blood was rejected.

The lipids were extracted from the serum with ethanol–ether (3:1, v/v), essentially as outlined by Bloor, Pelkan & Allen (1922). Cholesterol was precipitated as digitonide and determined colorimetrically, as described by Sperry & Webb (1950).

RESULTS

Natural rations. Free cholesterol in the serum of young adult rats consuming the stock diet (Master Fox Chow, Toronto Elevators, Ltd.) supplied in our laboratory varied from 8 to 18 mg./100 ml. The average for 18 female rats was 12·0 mg. with a standard deviation of ± 1·7 and for 29 male rats was 12·6 mg. ± 2·5. Bound cholesterol varied from 40 to 75 mg./100 ml., two-thirds of all values falling between 48 and 66. These limits are indicated by a vertical arrow in Fig. 1 and by dotted horizontal lines in Figs. 2 and 3. The average bound cholesterol in the serum of females was 56 ± 9 and in males was 57 ± 11.

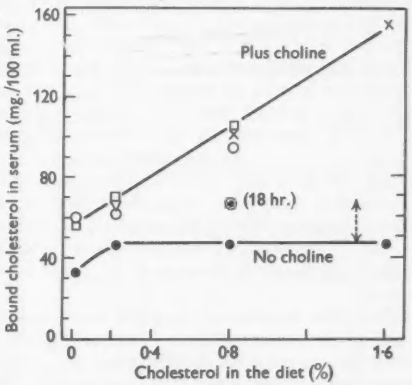

Fig. 1. Effect of dietary cholesterol on bound cholesterol in blood serum during the absorptive state. Groups of ten rats fed test diets for 3 weeks. All blood samples taken 2–4 hr. after food dishes removed, with the exception of one group (⊙) (choline 0·48%, no food available for 18 hr.). No choline (●); choline supplements (given as chloride in amounts 1·15 times values mentioned): 0·04% (▽), 0·08% (○), 0·16% (□), 1·28% (×). Vertical arrows represent limits within which two-thirds of all values normally fall in rats of our colony.

Preventive studies

Purified diets without added cholesterol. Blood samples were collected 2 to 4 hr. after removal of food dishes from the cages. The bound serum cholesterol of rats fed for 3 weeks on the basal (choline-free, cholesterol-free) purified ration was 32 ± 9 mg./100 ml. In rats fed the same diet containing 0·08% choline the bound cholesterol was 61 ± 6 mg. and doubling the amount of choline did not cause any further increase (57 ± 6 mg.), i.e. the presence of choline appears necessary in the diet to maintain the serum cholesterol within the normal range.

It may be mentioned that we have observed values for bound cholesterol as low as 10–12 mg./100 ml. serum in rats maintained for longer periods (6 months or more) on severely hypolipotropic, low protein diets. In these animals, liver damage was pronounced and in many of them cirrhosis of moderate severity was present.

Purified diets with added crystalline cholesterol. The data shown in Fig. 1 were obtained in a preventive experiment after the rats had been on the cholesterol-containing diets for 3 weeks. Blood samples from rats of all groups but one were collected in mid-morning 2–4 hr. after the food dishes had been removed from the cages. In one group (cholesterol 0·8%, choline 0·48%) blood was collected 18 hr. after removal of food. In the animals given choline a distinct relationship may be seen (during the post-prandial period) between the percentage of cholesterol in the diet and the average concentration of bound cholesterol in the serum. Because of the superposition of the points, it is impossible to show in Fig. 1 all the values determined, e.g. with 0·8% of cholesterol in the diet, the average bound serum cholesterol with different supplements of choline (0·32, 0·64 and 1·28%, respectively) was the same, viz. about 100 mg./100 ml. Thus, under these conditions considerable variation in the choline content of the diet, above the undetermined minimum, does not affect the cholesterol content of serum.

Data for the effects of betaine are not shown in Fig. 1, since the points would be superimposed on those already present. With 0·2% cholesterol in the diet and betaine equivalent to 0·16% choline the range of bound serum cholesterol was 51–79,

mean 62, S.D. ± 8; twice as much betaine gave similar values (61–77, mean 67 ± 7). At this dietary level of cholesterol supplementary inositol (0·32 %) had no effect on bound serum cholesterol, values found ranging from 23 to 38 (mean 30 ± 6).

Data in the previous paper (Ridout *et al.* 1954) showed that when excessive amounts of cholesterol are ingested, even large doses of choline are unable to prevent considerable deposition of esters in the liver: when 0·8 % cholesterol was in the diet, the amounts of cholesteryl esters that accumulated in the liver during 3 weeks with different amounts of dietary choline (0·08, 0·16 and 1·28 %) were, as percentage dry, fat-free liver, 10·3, 7·3 and 4·6, respectively, yet the sera from these different groups of animals contained essentially the same average concentrations of bound cholesterol. The levels in these sera are not related to the amounts of esters present in the liver. Obviously, the variations in the concentration of bound serum cholesterol shown in Fig. 1 must have another explanation. In all groups but one, blood was taken while some food may have still been present in the digestive tract. When the blood was not collected until 18 hr. after removal of food, the concentration in the serum was found to have returned practically to the normal range (mean value 68 mg./100 ml. as compared with about 100 mg. at 2–4 hr. after removal of the food). This observation seems to relate the concentration of cholesterol in the serum to the amount of cholesterol being absorbed from the gut, when choline is present in the food.

The influence of dietary choline on serum cholesterol was therefore examined further under two different conditions: (1) during absorption of cholesterol, with a much smaller deposition of cholesteryl esters in the liver; and (2) in a curative study, when there was no cholesterol in the diet but a large accumulation of esters was present in the liver.

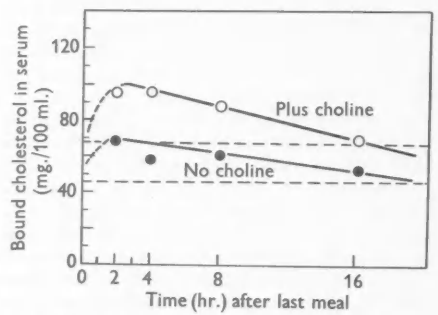

Fig. 2. Effect of absorptive state on bound cholesterol in serum. Dotted horizontal lines show limits within which two-thirds of values normally fall in our colony. Groups of ten rats fed diets containing 0·8 % cholesterol for 48 hr.; food dishes removed and blood samples collected after intervals shown. No choline (●), choline 0·32 % (○).

The first experiment involved two groups of rats fed the basal diet supplemented with 0·8 % cholesterol; those in one group got the hypolipotropic diet, the others were given the same food containing 0·3 % choline. The animals were offered these diets for 48 hr. and then ten rats from each group were killed 2, 4, 8 and 16 hr., respectively, after removal of food. When the diet contained choline, the bound serum cholesterol increased considerably between 2 and 4 hr. after a meal containing this excessive amount of cholesterol (Fig. 2); the level in the serum returned to normal in 16–24 hr. In the absence of choline there was a suggestion of a postprandial increase but the values scarcely left the normal range (69 mg./100 ml. at 2 hr. and 53 mg./100 ml. at 16 hr.). Analyses of the livers of the animals given choline revealed that a small accumulation of esters (0·4–0·6 % fresh liver) had occurred (this concentration was about 5 times normal compared with about 20 times normal in the previous experiment). Unfortunately, enough bound cholesterol was present in these livers so that mobilization rather than absorption might account for the peak. However, in the curative experiments now to be discussed the absence of any peak when the diet lacks cholesterol and the liver contains a large deposit of cholesteryl esters would seem to exclude mobilization as an important factor in the postprandial elevation of serum cholesterol.

Curative studies

Blood was collected from rats during two curative experiments after a cholesterol-rich diet had been fed for a preparatory period of 3 weeks to produce 'cholesterol fatty livers'. Cholesterol was determined in the serum of ten rats at this time. Blood was taken 2–4 hr. after removal of food dishes. The bound serum cholesterol varied from 23 to 62 mg./100 ml. (average 41). Cholesterol was removed from all diets during the curative phase; lipotropic supplements were incorporated in some of the rations.

In the first experiment half the rats were continued on the basal ration and half were given the diet supplemented with choline (0·48 %); groups of animals were killed after 1, 2, 4, 7 and 14 days, respectively, on the test diets. Within 24 hr. after transfer to the (cholesterol-free) ration containing choline, the bound cholesterol in the serum changed to a higher level (41–77 mg./100 ml., average 57) and remained there (on the 2nd day at 64, on the 4th day at 58, on the 7th day at 61 and on the 14th day at 68 mg./100 ml.). The corresponding average values were distinctly lower (43, 48, 44 and 41 mg./100 ml., respectively) in rats maintained on the

cholesterol-free basal diet for the same periods. The prompt restoration of the bound serum cholesterol to normal by choline is noteworthy. In these rats there was no dietary cholesterol in the chyme but there was an excessive accumulation of cholesteryl esters in the liver. No postprandial increase in serum cholesterol was observed in this experiment. Indeed, in this short-term curative study the bound cholesterol in the serum of rats not getting choline was on the average slightly below normal (41–48 mg./100 ml.) while in those given choline the values were in the upper portion of the normal range (57–68 mg./100 ml.). These findings suggest that choline may have a limited mobilizing effect, viz. that it acts to maintain the bound serum cholesterol within the normal range. Thus the large postprandial increases seen in the previous experiment were more likely due to some effect of choline on absorption or utilization, rather than on mobilization.

In subsequent experiments blood samples were not collected until 18 hr. after the removal of food, although the absence of cholesterol from the food may make the long interval unnecessary in curative studies; this procedure was followed, however, for the sake of uniformity and to minimize the effects of absorption.

During the long-term curative experiment, in which rats were fed the cholesterol-free test diets for periods of 14, 28, 42 and 70 days, respectively, the bound serum cholesterol fell progressively (Fig. 3) in the animals fed the choline-free basal ration. In other rats, when choline or betaine was restored to the diet, the concentration of bound cholesterol in the serum returned to the upper region of the normal range and remained there throughout the test periods. Inositol had no such effect. Vitamin B_{12} had a distinct effect in restoring the serum cholesterol; in the single set of data available (at 42 days) the value was within the normal range.

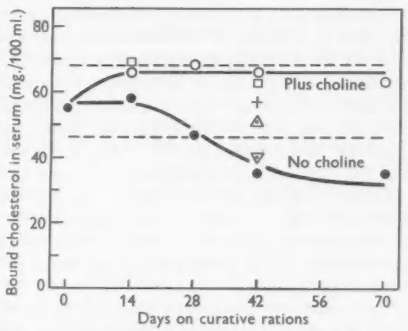

Fig. 3. Effect of lipotropic substances on bound serum cholesterol at different stages of a curative experiment. Groups of ten rats with 'cholesterol fatty livers' fed test diets for periods shown. No lipotropic supplement (●), choline 0·32% (○), betaine HCl 0·42% (□), vitamin B_{12} 10 μg./100 g. (△), inositol 0·32% (▽), choline 0·32% plus inositol 0·32% (+). Horizontal dotted lines represent normal values.

To give some idea of the variability of the concentration of bound cholesterol in the sera under these conditions, the ranges and means with standard deviations are given for the animals that had been fed the cholesterol-free test diets for 42 days: hypolipotropic basal diet, 24–44, mean 35 ± 8; plus choline, 55–70, mean 66 ± 6; plus betaine, 53–74, mean 63 ± 8; vitamin B_{12}, 46–60, mean 51 ± 5; inositol, 36–45, mean 40 ± 4; choline plus inositol, 46–73, mean 57 ± 14 mg./100 ml.

Mention may be made of the fact that a striking parallelism was observed between the behaviour of bound cholesterol and of serum phospholipids in the few cases where the latter were determined.

DISCUSSION

The concentration of cholesterol in the serum at any instant is the resultant of a number of interacting processes: (1) absorption, (2) mobilization, (3) synthesis, (4) degradation, and (5) excretion. Some factors which affect the regulation of blood cholesterol have been mentioned by Byers, Friedman & Rosenman (1952). In a review of lipid metabolism and atherosclerosis, Gould (1951) concluded that the normal plasma cholesterol content appears to be regulated chiefly by the liver. It is well known to clinicians that in patients with fatty infiltration of the liver or with cirrhosis, cholesteryl esters in the serum are low (Peters & Van Slyke, 1946).

The widely recognized value of the lipotropic agents in maintaining normal liver function may explain why in preventive experiments choline checks the fall in serum cholesterol which would otherwise occur. In the curative studies choline or betaine brought the serum cholesterol up to the normal value. In these curative studies the serum cholesterol was restored to normal within 24 hr. by choline, although as long as 10 weeks or more may be required to restore the cholesteryl esters in liver lipids to normal. The significance of this is not yet understood.

The experimental conditions adopted in other laboratories for the investigation of factors affecting serum cholesterol have differed in various ways from those followed in the present work, yet there is an essential agreement that supplementary choline increases the serum cholesterol in omnivorous animals fed cholesterol-containing hypolipotropic diets (Castro-Mendoza, Jiménez Díaz & Vivanco, 1947; Handler, 1948). We prefer to express this idea by saying that choline prevents the fall in serum cholesterol which is observed in rats fed hypolipotropic diets. In a series of experiments in which

cholesterol absorption was enhanced by incorporating bile salts in the diet, Weiss, Marx & Marx (1952) showed that supplementary choline plus inositol more than doubled the concentration of serum cholesterol. These high values may be postprandial peak values rather than sustained levels.

Others have noted that in dogs maintained on hypolipotropic diets the cholesteryl esters of the plasma were decreased, and that restoration of choline to the rations brought the ester concentration back towards normal (McKibbin, Thayer & Stare, 1944; McKibbin, Ferry, Thayer, Patterson & Stare, 1945). Burns & McKibbin (1951) also noted a progressive fall in plasma cholesterol in dogs during prolonged feeding of a hypolipotropic diet and reported that vitamin B_{12} ameliorated the condition. In the present study, a similar observation was noted with vitamin B_{12} in rats.

The failure of inositol to affect the serum cholesterol may be related to its inability to prevent the accumulation of cholesteryl esters in the liver or to hasten their removal from it. This is another example of a difference between the effects of inositol and of the substances (choline, betaine, methionine and vitamin B_{12}) referred to collectively as the lipotropic agents. It may be recalled that inositol also fails to protect weanling rats against the haemorrhagic kidney lesions induced by a deficiency of the lipotropic agents (Best, Lucas, Patterson & Ridout, 1946; Handler, 1946).

One point which our studies have emphasized is the importance of the time interval between the last meal and the collection of the blood sample. Under our experimental conditions, it was only when both an excessive amount of cholesterol and some choline were present in the diet that the concentration of bound serum cholesterol was significantly elevated in the first few hours after ingestion of the food. The magnitude of this elevation was related to the amount of cholesterol ingested and was apparently independent of the quantity of choline (or betaine) above some minimum. It takes much longer (18–24 hr.) for the postprandial increase in serum cholesterol to return to normal after a meal than it does for that of some of the other blood components, such as glucose or amino acids. This postprandial increase in the presence of choline may be brought about by a direct effect on the absorption process, by a secondary one on this process exerted perhaps through an effect on bile formation, or by some more obscure effect on utilization or storage.

Some workers have contended that an increase occurs in serum cholesterol after cholesterol feeding; others have insisted that dietary cholesterol has at most only a moderate influence on the level of cholesterol in the blood. The presence or absence of lipotropic agents in diets containing cholesterol and the state of absorption at which the blood samples were taken may partly explain the contradictions. The extent to which differences in the response of different species may also be a factor has not been fully explored. The failure of many investigators to record the interval of time between the last meal and collection of the blood samples is unfortunate since variations in this interval could give results leading to quite different impressions as to the influence of choline on the serum cholesterol. In order to establish more precisely these time relationships we plan to repeat parts of the study, and to feed the final meal by stomach tube.

Much of the experimental work in this field has been done under extreme conditions in which excessive amounts of cholesterol (1 or 2%) have been added to natural diets which would contain variable amounts of the ubiquitous lipotropic substances. Diets containing 1 or 2% of cholesterol (as percentage of dry, total solids) are probably never ingested under natural conditions. Actually, values exceeding 0·3% cholesterol are rarely encountered in rations ordinarily consumed. Keys (1949) has reported the habitual cholesterol intakes of 312 American men in a comfortable economic situation. The average weekly intake of the most extreme group in his series was 4·8 g. of cholesterol. Pihl (1952) has calculated the average daily intake of cholesterol in an average Norwegian diet to be about 550 mg. These values are in good agreement. Assuming that the dry solids in the diets would average between 500 and 700 g. per day, cholesterol would rarely exceed 0·1% of the total dry solids. The much more limited data reported for the cholesterol intake of nurses in a Canadian hospital (Ridout, Lucas, Patterson & Best, 1952) agree moderately well with this percentage of cholesterol in an average diet. Thus, many of the experimental diets used in studies of atherosclerosis have contained at least 10 to 20 times the amounts of cholesterol usually found in rations eaten by man.

In rats consuming diets containing lipotropic factors, it was only with excessively high intakes of dietary cholesterol (greater than 0·4%) that a marked postprandial elevation in bound serum cholesterol occurred. At reasonable intakes, corresponding to those in average rations consumed by man, the increase after meals was so small that the peak value did not go beyond the upper limits of the normal range.

Hypercholesterolaemia was observed in dogs fed a high fat diet containing cholesterol to which lecithin of adrenal origin had been added (Corwin, 1938) and also in human subjects and dogs given diets containing egg-yolk powder, which is rich in cholesterol and lecithin (Okey & Stewart, 1933; Steiner & Domanski, 1941; Glomset & Bollman, 1943; Messinger, Porosowska & Steele, 1950). Messinger *et al.* (1950) comment that the amount of

egg-yolk necessary to produce this effect is well above that consumed in any ordinary diet. Comparable effects were not produced by feeding diets rich in fat to which purified cholesterol had been added (Turner & Steiner, 1939), a fact which led Steiner & Domanski (1941) to postulate that the hypercholesterolaemia might be related to the lecithin contained in the egg-yolk. To test this hypothesis Steiner & Domanski (1944) added 'soya lecithin' (25 g. daily) to the hospital diet of eight patients; in every case a decrease occurred in the level of serum cholesterol. Others have noted a similar decrease when 'soya lecithin' was added to the diet (in patients with xanthomatosis, Gross & Kesten (1943) and Adlersberg & Sobotka (1943); Kesten & Silbowitz (1942) found that the feeding of 'soya lecithin' inhibited the hypercholesterolaemia usually observed in rabbits fed diets rich in cholesterol). The soybean product sold under the trade name of 'soya lecithin' contains only about 20 % of lecithin; its residual composition in approximate percentage, is 'cephalins' 20, oily glycerides 30, carbohydrates 10, inositol-containing complexes 15, and phytosterols 2, which is obviously a very different mixture from the egg-yolk and adrenal lecithin fed by the workers previously mentioned, who observed the opposite effect. To add to the confusion in this field Delevett & Bruger (1948) reported that 'soya lecithin' (either crude or oil-free) failed to reduce the serum cholesterol in four patients with xanthomatosis.

Because of the frequency with which atherosclerosis occurs spontaneously in birds, the chicken has often been used in the study of factors concerned with the development and control of vascular lesions. Using this species Stamler, Bolene, Harris & Katz (1950) observed that choline plus inositol failed to exert a prophylactic effect on the hypercholesterolaemia produced by feeding diets enriched with cholesterol. Indeed, their data show that during the first weeks of the experiment, addition of lipotropic factors actually aggravated the hypercholesterolaemia. It should be noted that the basal ration they adopted was made by adding cholesterol to a commercial chick starter mash containing mixed grains, dried buttermilk, soybean meal, meat and bone scraps, etc. Since these commercial rations are designed to be adequate with respect to all nutriments required by the growing chick, it may be assumed that essentially optimum amounts of choline and inositol were present in the basal ration. In the light of this and of our findings in rats, it is difficult to interpret their observation that adding more of the lipotropic agents caused such a remarkable elevation of the cholesterol in plasma.

Hypercholesterolaemia and atherosclerosis have been produced in Cebus monkeys by Mann, Andrus, McNally & Stare (1953) by feeding purified diets containing 5 % cholesterol. The hypercholesterolaemic response appeared to depend on a diet low in methionine and upon the presence of choline. In these experiments the hypercholesterolaemia was not merely postprandial but was sustained. The difference in response of different species to dietary cholesterol and choline may be much greater than is generally recognized.

During recent years there has been widespread use in North America of lipotropic substances in patients with atherosclerosis. There are, however, very few carefully controlled clinical studies. Many experimenters and clinicians have complicated the situation and rendered the interpretation of their results difficult by adding choline to natural diets of variable but unknown choline content. Sometimes this has been done deliberately. Firstbrook (1950), for example, reported that even large doses of supplementary choline had no effect on the total cholesterol of the blood of rabbits fed a commercial ration supplemented daily with 1 g. of crystalline cholesterol. It would have been clearer to the uninitiated if it had been emphasized that the effect of very large doses of supplementary choline were being tested and that no study of the effect of basal diets low in lipotropic factors was made. Davidson (1951) has reviewed this field and concluded that despite individual positive reports, consideration of all the available data indicates that there is no general agreement that choline or inositol has any specific influence upon atherosclerosis or upon the serum cholesterol in man or in experimental animals.

Katz & Stamler (1953) have pointed out that practitioners are being persuaded to prescribe expensive lipotropic preparations for the prophylaxis and therapy of human arteriosclerosis despite the inconsistency of the results and the paucity of controlled clinical data: 'From both scientific and socio-economic considerations, therefore, it is essential that the actual effects of lipotropic factors on atherosclerosis be clarified.' One of us (Best, 1950), evaluating the evidence for the therapeutic use of the lipotropic agents in atherosclerosis stated: 'This claim for choline has no firm basis in fact and may serve as a false pathway which diverts energy from both the profitable study of atherosclerosis and the real realms of choline action'. It is, of course, possible that *new* approaches may reveal a relationship of the lipotropic agents to atherosclerosis. Some suggestive leads are now being explored in our laboratory (e.g. Hartroft, Ridout, Sellers & Best, 1952).

SUMMARY

1. Free and total cholesterol have been determined in the blood sera of young adult rats maintained on natural and on purified diets. In rats of

our colony fed the stock ration, the mean total cholesterol is 70 mg./100 ml. (S.D. ± 11) of which 12 mg./100 ml. are in the free form. The values found in males and in females did not differ significantly.

2. The time, with respect to the last meal, at which the diet contains lipotropic agents and excessive amounts of cholesterol. The postprandial elevation in bound serum cholesterol was related to the concentration of cholesterol in the ration. Increasing the lipotropic agents (choline) above an undetermined minimal amount produced no further effect. The elevated serum cholesterol values returned slowly to normal after about 18–24 hr. In the absence of choline the postprandial increase (if any) was very slight even in the presence of large amounts of cholesterol in the diet.

3. In rats fed purified hypolipotropic diets, the bound serum cholesterol fell progressively as the experiment continued.

4. In preventive studies choline and betaine maintained the serum cholesterol within the normal range but inositol failed to prevent the characteristic fall.

5. In curative studies replacement of choline in the diet promptly restored (within 24 hr.) the concentration of serum cholesterol to normal; betaine also was able to restore and maintain the serum cholesterol; vitamin B_{12} exerted a similar effect; inositol did not alter the values observed in the rats continued on the basal ration.

6. Our experimental findings do not support the claims that lipotropic substances reduce the concentration of bound cholesterol in the serum. They do indicate that the same substances which prevent or cure fatty livers are also involved in the control of the cholesterol level in the serum.

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Iron Metabolism in the Laying Hen

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The laying hen excretes relatively large quantities of iron in the eggs. In our experience, a weekly output of 6 mg. Fe is not uncommon, and a good layer may lose 150–200 mg. in a year. This loss, which is not suffered by the non-laying bird, is so great that interesting changes in the processes of iron metabolism must precede or accompany the onset of laying. This paper presents the results of some investigations in this field.

Widdowson & McCance (1948) showed that the domestic fowl resembles many mammals in that the liver of the female contains more iron than that of the male. The effect, however, is manifest some weeks before the onset of laying, and the quantity of iron involved is small compared with that in the eggs. It is well known that the blood of cocks contains more haemoglobin than that of hens, while Cook & Harmon (1933), Maughan (1935) and Harmon (1936) all reported that blood haemoglobin was lower in the laying than in the non-laying hen. Cook & Harmon (1933) and Harmon (1936) went further, and claimed an inverse relation between blood haemoglobin and egg production during the laying season. Their results, however, were quoted in terms of mean haemoglobin and total egg production for an entire flock, and might therefore be explained by changes in the numbers of laying and non-laying birds. All these findings stand in opposition to those of Dukes & Schwarte (1931) and Winter (1936), who found no relation between egg production and blood haemoglobin. A re-investigation seemed necessary, and since some of the findings suggested that the demand for iron for the eggs might create an iron deficiency in the laying bird, serial determinations of total blood iron and plasma iron were undertaken in a small flock of birds throughout a laying season. All eggs were also analysed for iron.

In a separate experiment pullets were killed at intervals over the period during which laying commenced, and the livers, spleens and kidneys analysed for total and non-haem iron.

METHODS

General. All birds were maintained at the Poultry Research Centre, Edinburgh. Brown Leghorns were used, and most were fed on the stock breeding diet. This contains a supplement of $FeSO_4$, and provides 5–6 mg. Fe per day. A few birds were fed for 2 months on the same diet without the iron supplement, but although this lowered the dietary intake of iron by some 20%, these birds behaved in all respects similarly to the others. No attempt has therefore been made to present the results separately.

The birds were trapnested to permit the collection of eggs from individual birds.

Blood specimens were drawn as required (usually 4–5 ml. every 3 weeks) from the alar vein into heparinized ('Liquemin' Roche) syringes. It was assumed that the loss of 1·2–1·5 mg. Fe in each blood specimen would be insignificant in comparison with the loss of 12–20 mg. in the eggs during the 3-week period.

Total blood iron was determined on 0·05 ml. specimens by the H_2O_2–sulphite–dipyridyl technique of Ramsay (1952), except that the final heating was continued for 15 min. to ensure full colour development, and the volume adjustment was made with water instead of ethanol to a total of 7·5 ml. Haemoglobin concentrations were calculated from the results by assuming that fowl haemoglobin resembles many mammalian haemoglobins in containing about 0·34% Fe.

Plasma iron was determined by the NH_2OH–dipyridyl technique of Ramsay (1953). As this method gives high results on perceptibly haemolysed specimens, it is important to note that none of the samples analysed showed any spectroscopically detectable trace of haemolysis. The technique was checked by making duplicate analyses on pairs of specimens taken almost simultaneously with different apparatus from the two wings of each bird. The difference between the two wings never exceeded 10%, and was usually much less.

Egg iron was determined by the NH_2OH–dipyridyl technique of Ramsay (1951). In the early stages of the work, and at intervals later, the eggs were analysed individually, but it was found that little information was lost and much time saved if the eggs laid by each bird were pooled weekly before analysis.

Liver, spleen and kidney iron. Suspensions which could be pipetted with wide-mouthed pipettes were prepared by cutting up the whole organ in 5 or 10 vol. of water and treatment of the mixture in a macerator (Townson & Mercer) in which there was no risk of contamination with traces of metal from the bearings. A final treatment was given in the glass homogenizer of Potter & Elvehjem (1936). The spleens were so small that it was possible to omit the preliminary maceration.

Total iron was determined on 0·5–1·0 ml. portions of the suspensions by the H_2O_2–sulphite–dipyridyl technique (Ramsay, 1952), but it was found necessary to extend the heating period to 60 min. when the tissues of adult fowls were analysed. This procedure gave results similar to those obtained by destruction of the tissue with H_2SO_4, $HClO_4$ and HNO_3 before neutralization, reduction and treatment with dipyridyl. The difference between the two techniques was never more than 5%.

The determination of non-haem iron in these tissues could not be successfully carried out by heating with dipyridyl in the presence of sulphite or NH_2OH. Numerous other reducing agents were tested, but always added haemoglobin gave a high result, although added haemin (perhaps because of its insolubility in the acetate buffers used) did not. The failure of sulphite to prevent haemoglobin interference in these tissue analyses is interesting in view of its successful use in the determination of plasma iron in haemolysed specimens (Ramsay, 1954). It was found, however, that if both tissue suspension and reagents were fully saturated with CO before mixing and heating, the interfering effect of added haemoglobin was abolished, but only if sulphite was used as reducing agent. The treatment with CO was suggested by the experiments of Legge & Lemberg (1941), who showed that the liberation of 'easily split iron' from blood or haemoglobin solutions was greatly decreased by preliminary exposure to CO. It was found advantageous to work at a fairly low pH, and the procedure finally adopted was as follows. Tissue suspension (0·5–1·0 ml.), after saturation with CO (prepared by dropping conc. formic acid into conc. H_2SO_4) was pipetted into a tube graduated at 7·5 ml., and treated with 1 ml. 0·5 M-Na_2SO_3, and 5 ml. M acetate buffer, pH 4·25, containing 2:2′-dipyridyl (0·2%). The composition of the reagents is not critical. Both reagents were saturated with CO just before use. The tube was heated in a boiling water bath for 30 min., cooled, and the volume made up to 7·5 ml. After filtration through an iron-free paper the optical density was measured against a complete reagent 'blank' in a Unicam S.P. 350 Spectrophotometer at 520 mμ. If the CO treatment was adequate, the precipitated protein remained pink throughout the heating period. A change of colour to brown at any stage betrayed the necessity for a repetition of the analysis, not only because of the possibility of interference by haem pigments, but also because the rate of formation of the ferrous dipyridyl complex was much slower when the CO treatment was incomplete. Table 1 shows that inorganic iron added to liver suspensions was satisfactorily recovered, while haemoglobin iron did not affect the results significantly. Moreover, the livers analysed appeared to contain 85–101% of the total iron in non-haem compounds. For these reasons it was felt that the method probably gave a good estimate of non-haem iron, and that no great error would be risked in applying it to spleen and kidney, where the proportion of non-haem iron was much less (usually 50–60%).

Table 1. *Recovery of inorganic and non-recovery of haemoglobin* Fe *added to liver suspension*

Non-haem Fe originally present (μg./tube)	Fe added (μg.)	Non-haem Fe found (μg./tube)
	Inorganic Fe added	
12·5	3·4	15·8
12·5	3·9	16·4
12·5	5·1	17·3
6·5	5·6	12·0
9·2	4·8	14·3
	Haemoglobin Fe added	
20·2	10·3	19·6
23·6	10·3	23·9

EXPERIMENTAL AND RESULTS

In a first experiment sixteen birds (one of which laid poorly, became repeatedly crop-bound, and was ultimately discarded) were observed from the commencement of the laying season at the end of December until the following autumn. All eggs were analysed and at intervals of 3 weeks blood specimens were used for the determination of total iron and plasma iron. Four of the birds were unfortunately not bled until just after they had commenced to lay, and it was only possible to follow eight of the birds until they ceased to lay in the autumn. This was because laying commenced, in different birds, between the end of December and the end of February, and ceased between mid-September and some time after the end of October, when it was necessary to terminate the experiment. The general trend of the results, however, was so uniform, and has been so completely borne out by later experiments, that these omissions would seem to have been comparatively harmless.

Plasma iron. Until about 10 days before a hen began to lay, its plasma iron concentration lay between 100 and 250 μg./100 ml., a range similar to that found in man and many other mammals. Shortly before laying commenced it rose to 500–900 μg./100 ml., and fluctuated (usually narrowly) within these high limits as long as eggs were regularly produced. Cessation of egg production

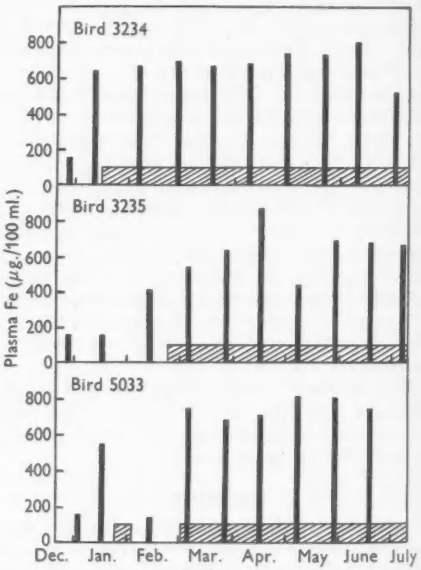

Fig. 1. Plasma iron in laying hens. The hatched areas indicate the periods during which each hen was laying.

was accompanied by a return to the original low level. No exceptional behaviour was detected. Fig. 1 shows three typical examples of the pre-laying rise and the maintenance of the high level during regular laying. Bird no. 5033 is interesting because a temporary cessation of egg production was matched by a fall in plasma iron. This diagram does not illustrate the post-laying decrease, but the eight specimens taken after the cessation of laying gave results of 117–191 μg. Fe/100 ml. with a mean of 137 μg./100 ml. Some figures from a later experiment, which show very clearly the relation of plasma iron concentration to the onset of laying, are given in Table 3.

Haemoglobin. Blood haemoglobin concentrations calculated from all the total iron determinations made before the middle of July are summarized in Table 2. The values were usually fairly constant during the laying period, and fluctuated by 5–10 % about a mean. In two birds only was there a definite tendency for haemoglobin to decline as the laying season progressed, but in 7 of 11 hens one or more pre-laying values was higher by 2–6 standard deviations than the mean of 8–10 values obtained during laying. Moreover, in two of the four in which no pre-laying decrease was noted the pre-laying specimen was taken only a day or two before laying commenced, and the plasma iron had already risen to the laying level. It seems very likely that in those cases the haemoglobin had already fallen to the laying level.

Egg iron. Although more than 2500 eggs were analysed, the results were of little intrinsic interest. The average iron content of all the eggs laid between March and June, when all the birds were laying regularly, was 0·88 mg./egg (range 0·76–1·07 mg.), rather less than other figures reported from this laboratory (Ramsay, 1950, 1951). The birds in all cases have come from the same Brown Leghorn strain, but the difference may result from the fact that the birds used in the present investigation were pullets, and laid eggs weighing only about 40 g.

Liver, spleen and kidney iron. As the investigations described above had shown changes in both plasma iron and blood haemoglobin to be associated with the onset of laying, it seemed of interest to find out whether there were also changes in the iron stores at this physiologically critical period. A series of pullets was therefore killed shortly before or shortly after they commenced to lay. Blood specimens were taken within a day or so of death, and the livers, kidneys and spleens removed for the determination of non-haem and total iron. An attempt was made to relate the results to the stage of ovarian development, which was described for present purposes in terms of the number and size of the yolks present. The results are summarized in Table 3.

DISCUSSION

Haemoglobin. The haemoglobin determinations made in this work are in fairly good agreement with the finding of Cook & Harmon (1933), Maughan (1935) and Harmon (1936) that the concentration of haemoglobin is lower in the blood of laying than of

Table 2. *Blood haemoglobin in laying hens*

Double and treble figures in the last column refer to two and three analyses on different occasions. Figures in parenthesis show the number of occasions on which determinations were made.

	Haemoglobin (g./100 ml.)		
	While laying		
Bird no.	Mean	S.D.	Before laying
3221	9·1 (9)	0·45	—
3234	8·6 (10)	0·46	10·3
3235*	8·9 (8)	0·60	11·5, 10·7, 12·0
3236*	8·8 (10)	0·60	—
3239	8·6 (8)	0·10	8·4, 8·4
5033	8·0 (6)	0·90	9·1, 9·9, 9·9
5080	8·7 (10)	0·61	—
5237	8·4 (8)	0·42	8·2
5272	8·5 (10)	0·35	—
5297	8·4 (10)	0·31	8·5†
5313	8·3 (8)	0·32	10·5, 10·3
5314	8·9 (8)	0·57	9·3†
5322	8·2 (10)	0·42	9·1†
5325	7·9 (9)	0·32	8·2, 10·3
5947	8·6 (9)	0·35	10·2, 10·3

* In these birds blood haemoglobin declined steadily from 9·7 and 9·9 g./100 ml. respectively to 8·0 g./100 ml. in 4 and 6 months after the onset of laying.

† These observations were made only very shortly before the onset of laying, and in each case the plasma Fe had already risen to the laying level.

Table 3. *Plasma liver, spleen and kidney* Fe *in hens during development of ovarian activity*

Figures in parentheses indicate the range of the individual observations.

Group	State of ovarian tract	No. of birds	Plasma Fe (μg./100 ml.)	Liver Fe (mg./organ)		Kidney Fe (mg./organ)		Spleen Fe (mg./organ)	
				Non-haem	Total	Non-haem	Total	Non-haem	Total
A	Immature	7	225	7·7	8·2	0·70	1·11	0·20	0·34
			(173–290)	(6·3–10·5)	(7·3–11·1)	(0·57–1·03)	(0·84–1·44)	(0·13–0·22)	(0·24–0·41)
B	A few yolks up to 6 mm. diam.	5	311	6·1	6·7	0·59	0·93	0·20	0·38
			(225–565)	(3·9–7·2)	(4·6–8·2)	(0·41–0·81)	(0·69–1·25)	(0·11–0·36)	(0·23–0·60)
C	Several yolks up to 15 mm. diam.	6	438	6·7	7·6	0·90	1·31	0·16	0·31
			(318–559)	(4·4–8·2)	(5·2–8·9)	(0·44–1·38)	(0·89–1·80)	(0·14–0·19)	(0·25–0·43)
D	First egg acquiring shell	1	711	9·2	9·4	0·70	1·05	0·22	0·33
E	Laying	5	768	5·7	6·0	0·82	1·27	0·14	0·27
			(670–942)	(4·1–7·9)	(4·3–8·9)	(0·59–1·09)	(0·91–1·57)	(0·12–0·16)	(0·24–0·31)

non-laying hens. The effect was smaller than that described by the earlier workers, the difference apparently being attributable to the fact that in their laying hens the blood haemoglobin fell to 6–7 g./100 ml., whereas our values were usually 8–9 g./100 ml., and rarely less than 7·5 g./100 ml. This, and the possibility disclosed in our experiments that some birds may not show the effect at all, may account for the contrary findings of Dukes & Schwarte (1931) and Winter (1936).

It is an attractive hypothesis that this decrease in haemoglobin is, perhaps through some endocrine intervention, dependent on the demand for iron for the eggs. The small decrease in circulating haemoglobin observed would, however, only provide 3–5 mg. iron for the eggs, and it is at least equally probable that the change in haemoglobin concentration reflects a change in plasma volume. We have made attempts to investigate this point by the determination of plasma volume with Evans Blue, but have encountered difficulties which seriously invalidate the results. The dye, for example, is very rapidly transmitted to the eggs.

The fact that the laying level of haemoglobin is maintained more or less constant in the face of continued excretion of iron in the eggs argues strongly against there being any degree of iron deficiency whatever.

Egg iron and plasma iron. When a comparison is made of egg iron with plasma iron, by plotting against one another the mean values of the two over the 4-month period March–June, when laying was most regular, the interesting fact emerges that there is a very definite relation between the two. This is shown graphically in Fig. 2. The correlation co-

Fig. 2. The relation between egg iron and plasma iron. The points were obtained by plotting the mean egg iron over 4 months against the mean plasma iron (determined every 3 weeks) during the same period. Fifteen birds were used. The straight line was calculated from the correlation coefficient $r = 0{\cdot}717$ ($P < 0{\cdot}001$).

efficient in our experiment was 0·717 ($P<0·001$). This finding is consistent with the view that the egg iron is directly derived from the plasma iron. Other statistical comparisons suggested that there was no relation between plasma iron and number of eggs or the total amount of iron excreted in the eggs, or between blood haemoglobin and mean egg iron, total egg iron, or number of eggs. The last of these comparisons supports the view that the relation found by Cook & Harmon (1933) and Harmon (1936) between blood haemoglobin and number of eggs, might have resulted from an unsound method of calculation. Increases in many blood constituents have been associated with egg production (most of the available information was tabulated and discussed by Romanoff & Romanoff, 1949), but it is believed that this is the first occasion on which a quantitative relation has been established between the plasma concentration of a substance and the amount present in the eggs.

Liver, spleen and kidney iron. Table 3 shows that the liver contains 8–10 times as much non-haem iron as the kidney, and that again 3–5 times as much as the spleen, which in birds is a small organ. In these hens the spleen weighed only 1–2 g. The liver thus contains the great bulk of the small store of iron carried by the hen. The livers of the non-laying pullets contained an average of 7·7 mg. non-haem iron while those of the laying ones contained 5·7 mg. The numbers of birds were too small for this difference to be considered significant, and in any case it is much less than the amount of iron (5–10 mg.) transferred by the laying birds to the eggs. A small number of analyses made at longer intervals after the onset of laying suggested that the non-haem iron in the liver may perhaps decrease slowly during the laying season.

It is interesting to note that the development of ovarian activity is accompanied by a large increase (from 25–35 g. to 45–60 g.) in the weight of the liver. The organ becomes pale and apparently fatty, although we have not checked this point by analysis.

Iron absorption. Even if it be assumed that the pre-laying decreases in blood haemoglobin and liver non-haem iron are real, and have the effect of providing iron for the eggs, these changes can provide iron for at the most some 6–8 eggs. There can be no doubt, therefore, that the demand for iron must be met very largely by an increase in the efficiency of iron absorption. It may be calculated from iron intake and egg iron that the laying birds in our experiments must have absorbed some 15–20 % of the dietary iron, in spite of the fact that the diet was rich in cereals, and hence in phytate, which might have been expected (Widdowson & McCance, 1942; McCance, Edgecombe & Widdowson, 1943) to exert an adverse influence on iron absorption.

SUMMARY

1. Determinations of egg and plasma iron over a whole season in fifteen birds showed a great elevation of plasma iron just before laying commenced. The high level persisted during the period of regular laying, and there was a high correlation ($r=0·717$, $P<0·001$) between mean plasma iron and mean egg iron.

2. Total blood iron determinations over the same period suggested that in many hens the onset of laying is preceded by a fall in blood haemoglobin. The lower level persists during the period of regular laying.

3. Determinations of total and non-haem iron in liver, spleen and kidney over the short period during which hens were coming into lay suggested that there may perhaps be a slight decrease in non-haem iron in the liver at this time, but that the quantity involved is small compared to that utilized for the eggs in the same period.

4. It is concluded that the demand for iron for the eggs must be met by an increase in the efficiency of iron absorption.

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The Biosynthesis and Metabolism of Betaines in Plants

2. THE BIOSYNTHESIS OF GLYCINEBETAINE (BETAINE) IN HIGHER PLANTS

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The synthesis of glycinebetaine may proceed by two possible routes: (*a*) by the stepwise methylation of glycine, with sarcosine and dimethylglycine as intermediates, or (*b*) by the stepwise methylation of ethanolamine to choline with subsequent oxidation of the latter base. In certain animal tissues there is abundant evidence of the formation of betaine by oxidation of choline, and this step would appear to be essential for the labilization of a methyl group of choline (Muntz, 1950). Since the work of Mann & Quastel (1937) and Bernheim & Bernheim (1938) who showed that rat-liver preparations are capable of oxidizing choline via betaine aldehyde to betaine, further investigation of the enzyme system has shown that the two components choline oxidase and betaine aldehyde oxidase are largely concentrated in the mitochondrial fractions of rat liver (Williams, 1952*a*, *b*). Although betaine is widely distributed in plants and occurs in high concentration in some families (Cromwell & Rennie, 1953*b*) little is known of the mode of synthesis and role of this compound in the metabolism of higher plants. Barrenscheen & Vályi-Nagy (1942) claim to have demonstrated a stimulation of betaine synthesis in homogenates of wheat seedlings to which glycine and methionine had been added, but repetition of these experiments in this laboratory failed to show any substantial conversion of glycine into betaine. In the present paper experiments are described in which possible precursors of betaine were infiltrated into leaves of *Beta vulgaris* L. and *Atriplex patula* L. with the object of determining the path of betaine synthesis. Preliminary experiments carried out by Cromwell & Rennie (1953*a*) on roots and leaves of seedling plants of *Beta vulgaris* from which homogenates were prepared seemed to show the presence of an enzyme which oxidized added choline to betaine. However, subsequent work showed that the activity of these homogenates was due to bacterial action.

EXPERIMENTAL

Materials and methods

Leaves of plants of *Beta vulgaris* L. and *Atriplex patula* L. in various stages of development were used in the following experiments. Two methods of introducing solutions of possible precursors of betaine into the leaf tissues were adopted. In the first method the leaves were placed with their petioles in tubes containing the appropriate solution and allowed to take up the solution for periods of 24 or 48 hr. The solutions were adjusted to pH 7 where necessary, and to minimize bacterial growth were sterilized by boiling for 2 min. Immediately before the experiment one half of the leaf blade was removed as a control and kept in a moist atmosphere under a bell jar under the same light and temperature conditions as the experimental half. On completion of the experimental period the remaining half leaf was removed from the midrib and both halves dried at 80° for 2 hr., powdered and stored in a desiccator until required for analysis. In the second method, the solutions, neutralized where necessary, were introduced into the leaf tissues by vacuum infiltration. After infiltration and evaporation of excess of water, the leaves were kept in a moist atmosphere under a bell jar during the period of the experiment. In one series of experiments, half leaves were infiltrated with water as controls and the remaining half was infiltrated with the experimental solution. In a second series of experiments on *Atriplex patula*, entire opposite leaves were used, one member of the pair being infiltrated with water and the opposite member infiltrated with the experimental solution. Before infiltration, the leaves were surface sterilized by immersion in a 1% (v/v) solution of 'Dettol' (Reckitt and Coleman Ltd., Hull), and water and solutions used for infiltration were sterilized by boiling for 2 min. On completion of the experiment the leaves were dried at 80°, powdered and stored in a desiccator until required for analysis.

Choline chloride, glycine and DL-methionine used in these experiments were purified by recrystallization. Dimethylglycine was used as the Na salt and was prepared by the method of Michaelis & Schubert (1936). Betaine aldehyde, (formylmethyl)trimethylammonium hydroxide, was prepared by the method of Voet (1929). Betaine was estimated by the method of Cromwell & Rennie (1953*b*) after removal, where necessary, of dimethylglycine and other amino acids by oxidation with Ag_2O. As the determination of betaine by the above method depends on measurement of the concentration of reineckate ion, the purity of the reineckate precipitates was checked by determination of the melting point and by paper chromatography. For chromatography a solvent system consisting of 3 vol. *n*-propanol and 1 vol. N acetic acid was used with the ascending method (Whatman no. 1 filter paper). The spots were detected by use of a 0·25% (w/v) solution of potassium bismuth iodide in ethanol or acetone to which one drop of glacial acetic acid had been added. It was found that the use of acetone as solvent gave a better definition of the spots than ethanol.

A saturated solution of bismuth triiodide in ethanol slightly acidified with 10% (v/v) H_2SO_4 (1 drop 10% H_2SO_4/10 ml. solution) also proved a satisfactory reagent for the detection of choline and the betaines. It is essential for good results that the papers should be thoroughly dried for at least 2 hr. at room temperature in a fume cupboard with forced draught before application of the above reagents. With the solvent system *n*-propanol–N-acetic acid, betaine has an R_F value of 0·39 and choline 0·54. Betaine reineckate dissolved in 75% (v/v) acetone can be placed directly on the paper but the acetone solution of choline reineckate must first be treated with dilute $AgNO_3$ solution to liberate the base before application to the paper.

RESULTS

The results of experiments on the uptake of solutions of choline through the petioles of leaves of *Beta vulgaris* are given in Table 1. In each experiment the uptake of choline has resulted in an increase in the betaine content of the leaves. The effect of infiltration of solutions of choline and other possible precursors of betaine into leaves of *Beta vulgaris* and *Atriplex patula* are shown in Tables 2 and 3. Infiltration of solutions of choline resulted in substantial increases in the betaine content of the leaves. The infiltration of betaine aldehyde did not stimulate the formation of betaine to the same extent as choline, but this may have been due to the lack of purity of the betaine aldehyde used in the experiments. Solutions of glycine and methionine in combination appeared to have little effect on the content of betaine, whereas solutions containing dimethylglycine in combination with methionine caused slight increases. The betaine content of leaves infiltrated with solutions of choline and kept during the experimental period in an atmosphere of nitrogen showed little variation from the controls.

DISCUSSION

The evidence presented by the foregoing experiments shows that of the possible precursors of betaine introduced into the leaf tissues of the experimental plants, choline is responsible for the greatest increase in the content of betaine and the inference is drawn that betaine is probably formed by the oxidation of choline. It will be convenient first to consider the effect of the infiltration of choline on the betaine content of the leaves of the annual plant *Atriplex patula*. The ability of the leaves to convert choline into betaine appears to depend to a considerable extent on the growth phase of the plants from which they are removed. Leaves from young plants and from old mature plants do not bring about conversion as readily as leaves from plants in the stage of active vegetative growth at mid-season. It is of interest to compare the production of betaine in the present experiments with the production of trimethylamine by the leaves of the closely related annual plant *Chenopodium vulvaria* L. The production of trimethylamine from choline by this plant has been shown to be greatest during the phase of active vegetative growth and as the plant reaches maturity the content of trimethylamine declines (Cromwell, 1950). The peak of activity for the transformation of choline into betaine or its breakdown to trimethylamine therefore coincides with the phase of active vegetative growth preceding maturity of the tissues, and it may be assumed that at this period the activity of the enzymes concerned is at its maximum. Alternatively, the synthesis of choline may reach a peak at this period and thus provide an optimum concentration of substrate for the enzymes responsible for the conversion of choline into betaine or breakdown to trimethylamine. In *Beta vulgaris*,

Table 1. *The effect of uptake of choline on the betaine content of leaves of* Beta vulgaris *by use of the half-leaf method*

Values in parenthesis refer to control experiments.

Source of leaves	Duration of experiment (hr.)	Half-leaf sample	Composition of solution	Betaine content (mg./g. dry wt.)	Δ (Betaine content) (mg./g. dry wt.)
Plants in vegetative phase	24	Experimental	Water	4·35 (4·00)	+0·35
		Experimental	Choline chloride (0·07 M)	3·28 (2·16)	+1·12
Plants in vegetative phase	24	Experimental	Water	7·03 (5·82)	+1·21
		Experimental	Choline chloride (0·036 M)	7·75 (5·70)	+2·05
Plants in vegetative phase	48	Experimental	Water	2·93 (2·89)	+0·04
		Experimental	Choline chloride (0·036 M)	3·88 (2·61)	+1·27
		Experimental	Choline chloride (0·07 M)	5·98 (3·31)	+2·67
Mature plants after flowering	48	Experimental	Choline chloride (0·036 M)	5·68 (2·91)	+2·77

Table 2. *The betaine content of leaves of* Beta vulgaris *after infiltration with possible precursor substances*

The half-leaf method was used throughout these experiments and the half leaves were either placed in darkness or given a period of illumination (approx. 16 hr.) equivalent to a normal day during the experimental period. All infiltrated solutions 0·02 M, except betaine aldehyde chloride, 0·05 M. Values in parentheses refer to the control experiments in which water was infiltrated.

Age of plants (months)	Duration of experiment (hr.)	Illumination	Solution infiltrated	Betaine content (mg./g. dry wt.)	Δ (Betaine content) (mg./g. dry wt.)
3	72	Dark	Choline chloride	14·20 (11·80)	+2·4
		Light	Choline chloride	15·00 (9·28)	+5·72
3	72	Dark	Choline chloride	14·00 (10·20)	+3·80
		Light	Choline chloride	13·74 (7·17)	+6·57
3	48	Light	Choline chloride	6·87 (4·48)	+2·39
3	72	Dark	Glycine + methionine	10·10 (9·80)	+0·30
		Light	Glycine + methionine	8·75 (9·85)	−1·10
3	72	Dark	Dimethylglycine + methionine	9·56 (9·07)	+0·49
		Light	Dimethylglycine + methionine	12·00 (11·30)	+0·70
1–2	24	Light	Betaine aldehyde chloride	9·50 (8·50)	+1·0
1–2	24	Light	Choline chloride	16·20 (13·40)	+2·80
		Light	Betaine aldehyde chloride	15·70 (13·80)	+1·90
1–2	24	Light	Betaine aldehyde chloride	6·97 (5·92)	+1·05
1–2*	24	Light	Choline chloride	3·47 (3·03)	+0·44

* Leaves kept in atmosphere of nitrogen during experimental period.

Table 3. *The betaine content of leaves of* Atriplex patula *after infiltration with solutions of choline chloride*

Half leaves (Expts. 1–6) and entire leaves (Expts. 7, 8, 9) were infiltrated with 0·02 M choline chloride and during the experimental period were either placed in darkness or given a period of illumination (approx. 16 hr.) equivalent to a normal day. Values in parentheses refer to the control experiments in which water was infiltrated.

Expt.	Source of leaves	Duration of experiment (hr.)	Illumination	Solution infiltrated	Betaine content (mg./g. dry wt.)	Δ (Betaine content) (mg./g. dry wt.)
1	Plants in early vegetative phase	48	Dark	Choline chloride	10·10 (9·00)	+1·10
			Light	Choline chloride	9·50 (7·65)	+1·85
2	Plants in late vegetative phase	48	Dark	Choline chloride	9·60 (6·90)	+2·70
			Light	Choline chloride	11·23 (7·07)	+4·16
3		24	Dark	Choline chloride	4·08 (3·13)	+0·95
			Light	Choline chloride	6·91 (4·70)	+2·21
4		48	Dark	Choline chloride	6·52 (4·94)	+1·58
			Light	Choline chloride	9·04 (5·88)	+3·16
5		6	Light	Choline chloride	7·86 (6·52)	+1·34
6		8	Light	Choline chloride	7·40 (6·52)	+0·88
7		24	Dark	Choline chloride	10·76 (8·46)	+2·30
			Light	Choline chloride	10·70 (6·74)	+3·96
8	Mature plants after flowering	24	Light	Choline chloride*	13·22	+0·78
			Light	Choline chloride†	12·94 (12·44)	+0·50
			Light	Choline chloride	13·82	+1·38
9‡		24	Light	Choline chloride	3·86 (3·97)	−0·11

* 0·05 M solution. † 0·033 M solution.

‡ Leaves kept in atmosphere of nitrogen during experimental period.

a biennial plant, the infiltration of choline into leaves of plants in the active vegetative phase of growth stimulates the formation of betaine to a considerable extent. Experiments on etiolated wheat seedlings to be described in a later paper have shown that no conversion of choline into betaine takes place when choline or its precursors are fed to the seedlings. Young seedling leaves of *B. vulgaris* have a comparatively low content of betaine and a progressive rise occurs with increasing age of the plants during the vegetative period. Similarly, young seedling leaves of *Chenopodium vulvaria* contain very little trimethylamine. The suggestion is put forward that the production of betaine in seedling plants does not take place to a great extent but as the seedlings grow to the mature plant, the capacity for production of betaine increases to a maximum. Kirkwood & Marion (1951) have shown that choline methyl groups are not utilized by sprouting barley to form the methyl group of hordenine, and Dubeck & Kirkwood (1952) found that the choline methyl group is not a precursor of the *N*-methyl group of ricinine in seedlings of *Ricinus communis* grown in darkness. Byerrum & Wing (1953), however, have shown that in young plants of *Nicotiana rustica* the methyl carbon of choline can be transferred to give the *N*-methyl carbon of nicotine, and these authors have suggested that, as in animal tissues, the compound which acts as the methyl donor in *Nicotiana* is betaine and that choline must first be oxidized to betaine before its methyl carbon becomes available for *N*-methylation. This suggestion receives support from the fact that betaine is known to be present in the leaves of *Nicotiana* spp. (Barger, 1914). Similarly Dubeck & Kirkwood (1952) have suggested that the inability of choline to contribute to the methyl groups of hordenine and ricinine in seedlings of *Hordeum* and *Ricinus* may be due to lack of the enzyme necessary to carry out the oxidation of choline to betaine. However, etiolated seedlings of *Hordeum* contain small amounts of betaine (Bregoff, Roberts & Delwiche, 1953) which (if betaine acts as a donor of methyl groups) should be available for transfer of methyl carbon to hordenine. If therefore the betaine of barley seedlings is derived from choline, the methyl carbon of choline would be expected to make some contribution to the *N*-methyl group of hordenine. It is noteworthy that in both sets of infiltration experiments, exposure of the leaves to light during the experimental period resulted in a higher betaine content than in leaves kept in darkness. On the assumption that the intracellular concentration of oxygen would be higher in leaves in which photosynthesis is taking place than in leaves kept in darkness, the higher conversion of choline into betaine in leaves exposed to light could be regarded as evidence in favour of the view that betaine is formed by the oxidation of choline. Further evidence for oxidative conversion is given by the inhibition of betaine formation which took place when leaves infiltrated with choline were kept in an atmosphere of nitrogen during the experimental period and by the observation that infiltration of betaine aldehyde led to an increase in the betaine content of the leaves. Little or no evidence was obtained from the present experiments for the synthesis of betaine by the stepwise methylation of glycine.

Attempts were made to study the enzyme system involved in the conversion of choline into betaine in leaves by the preparation of tissue homogenates, but all preparations proved to be quite inactive even in the presence of added cytochrome *c* and various cofactors. It was concluded that breakdown of the cell structure resulted in the loss of essential components of the enzyme system or resulted in the production of inhibitors. It is hoped to carry out experiments on mitochondrial fractions of leaf tissues in an endeavour to make a further study of the enzyme system.

Preliminary experiments (Cromwell & Rennie, 1953*a*) which appeared to show that homogenates of roots of seedling plants of *Beta vulgaris* brought about the conversion of added choline into betaine were repeated and it was found that, despite the use of thymol as an antiseptic during the incubation period, bacterial activity was responsible for the conversion. When toluene was used in place of thymol no conversion of added choline took place either in homogenates of young roots of *Beta* or in homogenates of etiolated wheat seedlings. Homogenates sterilized by passage through a Seitz filter also failed to bring about the conversion of added choline into betaine. Accordingly, the suggestion made in an earlier paper (Cromwell & Rennie, 1953*b*) that betaine is synthesized in the root system of young seedlings of sugar beet and translocated to the shoot system is not supported by the results of experiments described in the present paper.

It has been stated that bacterial systems have provided no evidence for the formation of betaine from choline (Welch & Nicol, 1952) but work carried out in the present investigation has shown that certain Gram-negative bacteria, possibly belonging to the genera *Pseudomonas* and *Flavobacterium* isolated from non-sterile homogenates of wheat seedlings, are able to oxidize choline to betaine quite readily. Further work on the identification of these organisms is in progress.

SUMMARY

1. Experiments on the effects of infiltration of possible precursors of betaine into the leaves of *Beta vulgaris* and *Atriplex patula* have been described.

2. The infiltration of solutions of choline and betaine aldehyde resulted in a significant increase in the betaine content of the tissues.

3. Little variation from controls was observed when solutions of glycine in combination with methionine were infiltrated into the leaves.

4. When leaves infiltrated with solutions of choline were kept in an atmosphere of nitrogen during the experimental period significant increases in the content of betaine were not observed.

5. Leaves infiltrated with solutions of choline and exposed to light during the experimental period showed a greater increase in betaine content than leaves kept in darkness during the experimental period.

6. The conclusion is reached that the synthesis of betaine in the tissues of higher plants takes place largely as the result of the oxidation of choline.

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The Biosynthesis and Metabolism of Betaines in Plants

3. STUDIES ON THE BIOSYNTHESIS OF PRECURSORS OF GLYCINEBETAINE IN SEEDLINGS OF WHEAT (*TRITICUM VULGARE* VILL.)

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Evidence has been obtained that infiltration of choline into leaves of *Beta vulgaris* L. and *Atriplex patula* L. leads to an increase in the betaine content of the leaves and the inference has been drawn that betaine is formed in the tissues of plants by oxidation of choline (Cromwell & Rennie, 1954). It was therefore of interest to investigate the mode of synthesis of choline on the assumption that it is a direct precursor of betaine. The extensive literature on the biosynthesis of choline in animal tissues has been reviewed by Jukes (1947), and it is now well established that choline is formed by the stepwise methylation of ethanolamine in the tissues of the rat. In plants, however, the biosynthesis of choline has received little attention, and the object of the present work was to determine whether or not the synthesis of choline follows the same path in plants as in animals, the method being to feed wheat seedlings with possible precursors. Kirkwood & Marion (1951) have shown that when potassium formate labelled with ^{14}C is fed to sprouting barley, the labelled carbon appears in the methyl groups of choline and hordenine. These authors suggest that the formylation of amines followed by reduction is a general route for the synthesis of *N*-methyl groups in plants and animals. Recent work by Ahmad & Karim (1953) on the biosynthesis of choline in germinating seedlings of the chick-pea has shown that methionine, acetone and methanol, substances which give rise to choline methyl groups in the animal, also stimulate choline synthesis in the plant. Steensholt (1946) found that the methyl group of methionine was not utilized by etiolated wheat seedlings for the *N*-methylation of ethanolamine to choline. It is clear, therefore, that the problem of choline synthesis in plants as in animals involves a study not only of transmethylation reactions in which intact methyl groups are transferred, but also of the synthesis of 'biologically labile' methyl groups *de novo*.

EXPERIMENTAL

Materials and methods

Seeds of *Triticum vulgare* (vars. Atle and Fylgia) were used throughout these experiments. To hasten germination the seeds were treated with conc. H_2SO_4 for 1 min. and rapidly washed free from acid in a stream of tap water. Sterilization of the seeds was carried out by immersion for 5 min. in a solution containing 0·1% (w/v) $HgCl_2$ and 0·5% (v/v) 'Teepol' detergent (Shell Chemicals Ltd., London), followed by washing in sterile distilled water. Roux bottles (1 l. capacity) containing 80 g. of coarse quartz sand were used for germination and were fitted with cotton-wool air filters of the conventional pattern to enable the cultures to be aerated under sterile conditions. The appropriate culture solution (40 ml.) was placed in each of the bottles, the entire apparatus sterilized by autoclaving and allowed to cool in the aseptic cabinet. Each bottle was sown with 7·5 g. (dry wt.) of seed which was evenly distributed over the surface of the sand by holding the bottle in the horizontal position and imparting a vigorous swirling motion to the slurry of sand. The bottles were placed in the incubator at 19° and set at an angle of 10° from the horizontal to allow the culture solution to drain to the bottom of the bottle. After germination of the seed had taken place (1–2 days) the bottles were lowered to the horizontal position for the remainder of the growth period, thus allowing the culture solution to bathe the root system of the seedlings. The cultures were aerated daily for a period sufficient to allow of the complete removal of accumulated CO_2. For this purpose, the outgoing air stream was allowed to bubble through a solution of methyl red indicator and aeration continued until the indicator changed to orange-yellow. At the end of the growth period (6–7 days) the seedlings were removed from the bottles, freed from adhering sand grains by means of a coarse sieve, well washed in running water and finally dried in an oven with forced draught at 80°. After drying, the coleoptiles and roots were easily removed from the seeds by rubbing gently over a coarse-meshed sieve. The dried material was finely powdered in a mortar and stored in a desiccator until required for analysis. The culture solutions contained 0·0125% (w/v) of the substances used as possible precursors of choline and of methylating agents, either singly or in combination, and where necessary the pH of the solutions was adjusted to 7. Controls using distilled water were set up and each experiment was carried out in triplicate.

The sodium salt of dimethylglycine was prepared by the method of Michaelis & Schubert (1936). All other compounds used were commercial samples purified either by redistillation or by recrystallization. DL-Methionine was used as the methyl donor and formate was supplied as the potassium salt.

Estimation of choline

The dried plant material (0·1–1 g.) was extracted with methanol, following the method for the estimation of betaine (Cromwell & Rennie, 1953) to the stage of clarification with charcoal. At this stage the solution (5 ml.) was adjusted to pH 11–12 with 5% (w/v) NaOH, 5 ml. of a saturated aqueous solution of ammonium reineckate added and the tube placed in ice water for 1 hr. The precipitate of choline reineckate was filtered off on a chilled sintered glass crucible of medium porosity, the disk of which was covered with a thin layer of Gooch asbestos. The precipitate was washed with 5 ml. of cold ammonium reineckate solution and the filtrate and washings set aside for the estimation of betaine by the method of Cromwell & Rennie (1953). The precipitate of choline reineckate was washed with three portions of 2 ml. of *n*-propanol and finally with ether. A small glass rod was used to facilitate the complete removal of ammonium reineckate during the washing of the precipitate. The underside of the crucible was dried and the precipitate dissolved in 75% (v/v) acetone (4 ml.) and the solution collected in a test tube. The quantitative estimation of the choline reineckate was then carried out by the method of Marenzi & Cardini (1943). This method was slightly modified by adding the H_2SO_4 solution to the ethanolic solution of *sym.*-diphenylcarbazide before development of the colour.

RESULTS

The results of feeding etiolated wheat seedlings with possible precursors of choline and with compounds which might yield either intact methyl groups or which might contribute to the synthesis of labile methyl groups are given in Table 1. The possible precursor substances were either fed alone to the plants or in combination with the methylating compounds. Adequate precautions were taken to ensure that co-precipitation of unchanged methylated amines with choline did not take place as these substances were readily taken up by the seedlings. It was found that ammonium reineckate at pH 11–12 precipitates choline with no interference from methylethanolamine or dimethylethanolamine if these bases are present in admixture with choline. Analysis and melting point determinations were used to check the purity of the choline precipitates. Dimethylglycine is co-precipitated with betaine in acid solution and therefore was removed by oxidation with silver oxide prior to the estimation of betaine (Cromwell & Rennie, 1953).

The uptake of ethanolamine did not result in a significant increase in the content of either choline or betaine, but this base when fed in combination with formate caused a slight increase in the content of choline and a decrease in the content of betaine. The combination of ethanolamine with methionine led to a decrease in both choline and betaine values. When seedlings were allowed to take up methylethanolamine or dimethylethanolamine a remarkable stimulation of choline synthesis took place, and although the additional effect of the presence of methylating compounds varied somewhat, the combination of dimethylethanolamine with formate gave an increase of 172% in the choline value, but had comparatively little effect on the betaine content of the tissues. The combination of methylethanolamine with formate and/or methionine resulted in a loss of betaine. The uptake of glycine alone and in combination with methionine brought about a slight increase in choline values and a slight decrease in betaine. On the contrary, feeding with dimethylglycine alone or in combination with

Table 1. *The effect of possible precursor substances and substances yielding methyl groups on the biosynthesis of choline and betaine in etiolated wheat seedlings*

Experiments were carried out in triplicate and all analyses in duplicate. Choline and betaine values are expressed as mg./g. dry wt. Differences between experimental and control values are given on a percentage basis.

Possible precursor substance		Source of methyl groups: Choline	Source of methyl groups: Betaine	Methionine: Choline	Methionine: Betaine	Formate: Choline	Formate: Betaine	Methionine + Formate: Choline	Methionine + Formate: Betaine
No addition	Control	—	—	0·58	4·55	0·63	6·13	—	—
	(Mean variation)			(0·09)	(0·05)	(0·02)	(0·16)		
				+15	+15	+8	+2		
	Experimental			0·67	5·25	0·68	6·28		
	(Mean variation)			(0·07)	(0·22)	(0·05)	(0·28)		
Ethanolamine	Control	0·71	5·24	0·71	5·24	0·71	5·24	0·71	5·24
	(Mean variation)	(0·06)	(0·24)	(0·06)	(0·24)	(0·06)	(0·24)	(0·06)	(0·24)
		+15	+8	−11	−15	+20	−8	+14	+10
	Experimental	0·82	5·66	0·63	4·47	0·85	4·80	0·81	5·78
	(Mean variation)	(0·07)	(0·15)	(0·08)	(0·25)	(0·09)	(0·13)	(0·04)	(0·30)
N-Methylethanolamine	Control	0·70	6·49	0·70	6·49	0·70	6·49	0·70	6·49
	(Mean variation)	(0·07)	(0·11)	(0·07)	(0·11)	(0·07)	(0·11)	(0·07)	(0·11)
		+112	−1	+81	−27	+102	−27	+123	−29
	Experimental	1·48	6·41	1·27	4·74	1·41	4·76	1·56	4·63
	(Mean variation)	(0·13)	(0·13)	(0·25)	(0·11)	(0·09)	(0·20)	(0·09)	(0·25)
Dimethylethanolamine	Control	0·61	5·34	0·61	5·34	0·61	5·34	—	—
	(Mean variation)	(0·04)	(0·28)	(0·04)	(0·28)	(0·04)	(0·28)		
		+93	+6	+80	+18	+172	+3		
	Experimental	1·18	5·68	1·10	6·28	1·66	5·48		
	(Mean variation)	(0·15)	(0·55)	(0·14)	(0·27)	(0·19)	(0·21)		
Glycine	Control	0·69	6·23	0·69	6·23	—	—	—	—
	(Mean variation)	(0·06)	(0·19)	(0·06)	(0·19)				
		+22	−4	+16	−16				
	Experimental	0·84	5·97	0·80	5·24				
	(Mean variation)	(0·08)	(0·38)	(0·09)	(0·24)				
Sarcosine	Control	—	—	—	6·15	—	—	—	—
	(Mean variation)				(0·25)				
					+9				
	Experimental				6·68				
	(Mean variation)				(0·44)				
Dimethylglycine (Na salt)	Control	0·69	5·73	0·69	5·73	0·69	5·73	0·69	5·73
	(Mean variation)	(0·06)	(0·13)	(0·06)	(0·13)	(0·06)	(0·13)	(0·06)	(0·13)
		−12	+12	−26	+14	+4	+15	+7	−4
	Experimental	0·61	6·42	0·51	6·57	0·72	6·62	0·74	5·52
	(Mean variation)	(0·04)	(0·16)	(0·04)	(0·24)	(0·07)	(0·17)	(0·06)	(0·10)

methionine resulted in slight losses of choline but slight increases in the betaine values. The uptake of methionine and formate separately and in combination did not bring about a significant increase of either choline or betaine and sarcosine was ineffective as a stimulant of betaine synthesis.

DISCUSSION

Bearing in mind the difficulties attending the interpretation of results of feeding experiments on intact, actively growing seedlings, the work described in this paper may be regarded as giving support to the conclusion that choline is synthesized in the plant by the stepwise methylation of *N*-methylethanolamine and possibly by the stepwise methylation of ethanolamine. If the free choline in the tissues were to undergo immediate conversion into betaine or to take part in the synthesis of phospholipids, the difficulties of interpretation would be further increased. However, there appears to be little or no conversion of choline into betaine in the seedlings under the conditions of the experiments and phospholipids are not synthesized to any extent in the tissues of young, actively growing plants (Jordan & Chibnall, 1933; Byerrum & Wing, 1953). The experiments of Ahmad & Karim (1953) on the seedlings of the chick-pea have shown that feeding with DL-methonine stimulates the synthesis of choline to a considerable extent. In the present series of experiments, feeding of etiolated wheat seedlings with methionine or formate without the ethanolamines did not lead to a significant increase in the choline content of the tissues and it may be assumed that the effective concentration of endogenous methionine or 'formate' was not the limiting factor in choline synthesis. The uptake of ethanolamine alone and in combination with methionine and formate did not stimulate the synthesis of choline and in these experiments the apparent inability of etiolated wheat seedlings to use methionine for the methylation of ethanolamine is in agreement with the findings of Steensholt (1946). Similarly, mutant 34486 of *Neurospora crassa* is unable to utilize methionine for the methylation of ethanolamine (Horowitz & Beadle, 1943; Jukes, Oleson & Dornbush, 1945), and Jukes (1941) found that the chick is unable to utilize ethanolamine in the presence of methionine as a substitute for choline in a purified diet. However, when formate labelled with ^{14}C is fed to sprouting barley, the radioactive carbon appears in the methyl groups of choline (Kirkwood & Marion, 1951) and an incorporation of formate carbon into the methyl group of nicotine was reported by Brown & Byerrum (1952). More recently, Byerrum & Wing (1953) have shown that the carbon of the methyl groups of choline can be transferred to the *N*-methyl group of nicotine. It would therefore appear that in the tissues of *Nicotiana rustica* L. formate makes a contribution to the methyl groups of choline. From the experimental evidence available, the tentative deduction can be made that ethanolamine is not methylated to *N*-methylethanolamine by the transfer of an intact methyl group from methionine but requires for this reaction a one-carbon methyl group precursor, the carbon of which may be derived from formate, formaldehyde, methanol or acetone. The work of Arnstein (1951) on the biosynthesis of choline in the tissues of the rat has shown that L-serine is decarboxylated to ethanolamine and that methanol, formate, the β-carbon atom of serine and the α-carbon atom of glycine are precursors of choline methyl groups. Furthermore, Arnstein (1951) concluded that the rate of synthesis of choline methyl groups from the above precursors is slow compared with the rate of synthesis of the ethanolamine moiety and suggests that the apparently slow rate of methyl group synthesis is due mainly to an adequate supply of labile methyl groups in the diet. Similarly, if the rate of synthesis of labile methyl groups in etiolated wheat seedlings is slow, the lack of a significant stimulation of the formation of choline when ethanolamine is fed could be explained. Alternatively, ethanolamine when fed to etiolated wheat seedlings may undergo breakdown and therefore may be available to a limited extent only, for methylation to *N*-methylethanolamine. Weissbach & Sprinson (1953) have shown that ethanolamine is deaminated in the tissues of the rat with the formation of glycolaldehyde, which is oxidized through glycollic acid to glyoxylic acid which then becomes aminated to glycine. The synthesis of serine from glycine takes place through the incorporation of 'formate' and decarboxylation of serine results in the formation of ethanolamine. In the tissues of the rat, therefore, a cycle of reactions involving ethanolamine may take place. In the present series of experiments the stimulation of choline synthesis observed when *N*-methylethanolamine or dimethylethanolamine were fed to the seedlings may be ascribed to the ability of these compounds to accept an intact methyl group from methionine or other methyl donor. Moreover, the synthesis of the methyl group of methionine may be stimulated by formate, but on the other hand a one-carbon compound derived from formate may have been directly responsible for the methylation of these amines in the experiments in which formate was fed. When *N*-methylethanolamine and dimethylethanolamine were fed alone, the tissue content of choline showed a significant rise, thus lending support to the previous assumption that a substantially adequate endogenous supply of methyl donors was present in the tissues of the seedlings. In contradistinction to ethanolamine, it is unlikely that either *N*-methyl-

ethanolamine or dimethylethanolamine would be deaminated to any great extent and these substances would therefore be freely available for choline synthesis. The slight increase in choline synthesis observed when glycine was fed may be due to the ability of the α-carbon atom of glycine to act as a precursor of ethanolamine and the methyl groups of choline. The loss of betaine in some of these experiments is difficult to explain. The seed of wheat contains approx. 0·092 % of betaine (Guggenheim, 1940) and as the young seedling does not appear to synthesize betaine from choline or other possible precursors it is assumed that the betaine present in the seedling is translocated from the seed. As the fate of betaine was not determined in these experiments it is impossible to do more than suggest that on the analogy of the behaviour of betaine in animal tissues, this substance can also act as a methyl donor in plants. In experiments in which a loss of betaine was recorded, a methyl group derived from betaine might have made some contribution to the methylation of *N*-methylethanolamine and dimethylethanolamine to choline.

SUMMARY

1. Experiments on the effects of feeding possible precursors of choline to etiolated wheat seedlings grown under sterile conditions have been described.

2. The biosynthesis of choline was stimulated to a marked degree when *N*-methylethanolamine or dimethylethanolamine were fed alone and in combination with methionine and/or formate. *N*-Methylethanolamine or dimethylethanolamine used in combination with formate stimulated choline synthesis to a greater extent than the combination of these precursors with methionine.

3. The feeding of ethanolamine either alone or in combination with methionine and/or formate did not result in a significant increase in the choline content of the tissues.

4. The feeding of glycine alone and in combination with methionine brought about a slight increase in the choline content of the tissues but a slight decrease in the content of betaine. The feeding of dimethylglycine alone or in combination with methionine resulted in slight losses of choline but a slight increase in the content of betaine. A slight increase in the content of betaine was observed when dimethylglycine was fed in combination with formate.

5. Conversion of choline into betaine did not appear to take place in etiolated wheat seedlings under the conditions of the experiments. The possible significance of losses of betaine which occurred in some experiments is discussed.

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The Biosynthesis of Proteins

2. SYNTHESIS OF MILK PROTEINS BY THE GOAT

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In the first paper of this series (Campbell & Work, 1952) it was shown that the lactating rabbit synthesized milk protein mainly from the free amino acids of the blood. The distribution of radioactivity between casein and whey proteins showed, however, that if casein is synthesized entirely from free amino acids then a portion of the whey nitrogen must be supplied as bound amino acid, probably in the form of whole protein or peptide.

It has been shown recently (Askonas, Campbell, Humphrey & Work, 1954) that the immune globulin of rabbit milk is not synthesized in the mammary

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gland; thus, the presence of this protein in whey could account in part for the low radioactivity of whey protein as compared to casein. The quantity of immune globulin in milk is low and it seemed doubtful whether this protein alone could account for all the 'bound' amino acids of whey and, particularly, for the low specific radioactivity of whey serine as compared to casein serine after injection of radioactive glycine.

Since whey contains a complex mixture of proteins it was undesirable to make further comparison between casein and whey protein and it was decided to compare instead casein and β-lactoglobulin. It was not possible to obtain sufficient rabbit milk to permit isolation of the lactoglobulin fraction and the present series of experiments have therefore been made on a goat. A method for the crystallization of goat β-lactoglobulin is given in another paper (Askonas, 1954) along with details of its physical properties. In the present experiments milk was collected after injection of radioactive glycine, valine, methionine and lysine. Comparison of the specific radioactivities of the amino acids from casein and from β-lactoglobulin showed (as with the rabbit) a substantial difference between casein serine and lactoglobulin serine but no significant difference of specific radioactivity between the lysine, valine and glycine from casein and the corresponding amino acids from β-lactoglobulin. A preliminary account of some of this work has been published (Work, Campbell & Askonas, 1952).

MATERIALS AND METHODS

Animal. A British Saanen goat was used, 2 years old and in her first lactation period. Her milk yield was about 5 lb. daily.

Radioactive amino acids. Glycine was supplied as [2-^{14}C]-glycine by the Radiochemical Centre, Amersham; DL-[^{35}S]methionine and DL-[4-^{14}C]valine were obtained from the same source. DL-[1-^{14}C]lysine was kindly supplied by Dr Hunter (Arnstein, Hunter, Muir & Neuberger, 1952).

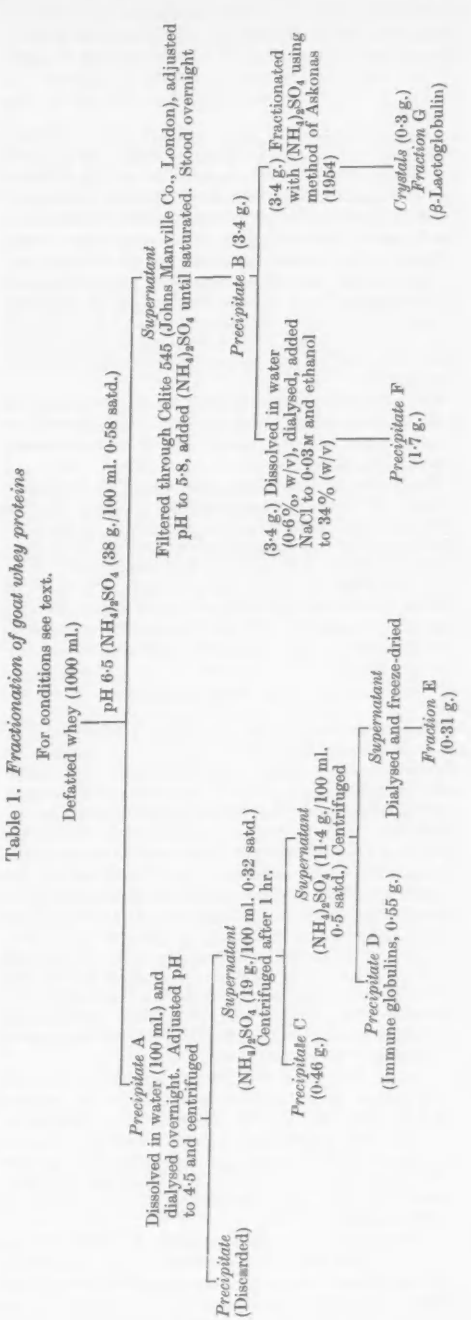
Administration of radioactive amino acids. A suitable quantity of amino acid was dissolved in isotonic saline and injected into the jugular vein within 1 hr. of the morning milking.

Collection of milk samples. When it was necessary to obtain the maximum yield of radioactive protein, the animal was milked by hand in the normal way until no more milk could be obtained; it was then given an intravenous injection of 5 i.u. of posterior pituitary extract (British Drug Houses Ltd.) and milked dry.

Fractionation of proteins from goat milk

Casein. Milk (1 l.) was centrifuged to remove fat, and the casein precipitated by slow addition of a mixture of N-HCl and N acetic acid (1:2, v/v) to the stirred solution until the pH was 4·5 (Van Slyke & Baker, 1918). The casein was centrifuged down, washed twice with water and the first washings were added to the whey fraction. The casein was

Table 1. *Fractionation of goat whey proteins*


For conditions see text.
Defatted whey (1000 ml.)
pH 6·5 (NH_4)$_2$SO$_4$ (38 g./100 ml. 0·58 satd.)
Precipitate A
Dissolved in water (100 ml.) and dialysed overnight. Adjusted pH to 4·5 and centrifuged
Precipitate (Discarded)
Supernatant
(NH_4)$_2$SO$_4$ (19 g./100 ml. 0·32 satd.)
Centrifuged after 1 hr.
Precipitate C (0·46 g.)
Supernatant
(NH_4)$_2$SO$_4$ (11·4 g./100 ml. 0·5 satd.) Centrifuged
Precipitate D (Immune globulins, 0·55 g.)
Supernatant
Dialysed and freeze-dried
Fraction E (0·31 g.)
Supernatant
Filtered through Celite 545 (Johns Manville Co., London), adjusted pH to 5·8, added (NH_4)$_2$SO$_4$ until saturated. Stood overnight
Precipitate B (3·4 g.)
(3·4 g.) Dissolved in water (0·6% w/v), dialysed, added NaCl to 0·03 M and ethanol to 34 % (w/v)
Precipitate F (1·7 g.)
(3·4 g.) Fractionated with (NH_4)$_2$SO$_4$ using method of Askonas (1954)
Crystals (0·3 g.) Fraction G (β-Lactoglobulin)

redissolved by suspension in water (2–3% protein) and dropwise addition of N-NaOH to pH 6·5. The solution was shaken with an equal volume of ether to remove residual lipid and the casein in the aqueous layer reprecipitated at pH 4·5, washed twice and freeze-dried. The yield was about 26 g.

Whey protein. The whey, adjusted to pH 5·5, was defatted by shaking with an equal volume of ether. The aqueous layer was freed from ether *in vacuo* at 30° and the proteins were fractionated with $(NH_4)_2SO_4$, following, with minor modifications, the procedure of Smith (1946) for bovine whey (see Table 1). Solid $(NH_4)_2SO_4$ (A.R.) was added at room temperature with continuous stirring and all mixtures were allowed 2–3 hr. to reach equilibrium unless otherwise stated. All precipitates (Table 1) were dissolved in the minimum amount of water, dialysed against running tap water and then for 48 hr. at 1° against distilled water and freeze-dried. The yields given in Table 1 represent an average for several experiments.

Electrophoretic analyses of whey fractions were carried out in a Tiselius electrophoresis apparatus (A. Hilger and Co., London) in various buffers ($I = 0{\cdot}1$) at 1° and at a concentration of 1–2% protein.

Purification of lactoglobulin. The crude β-lactoglobulin fraction (*B*, Table 1) was crystallized by the method of Askonas (1954). Fraction *B* was also purified by using the last step in the ethanol fractionation of Bain & Deutsch (1948). A 0·6% (w/v) solution of *B* was dialysed 24 hr. against distilled water, the pH was adjusted to 6·55 and 3 M-NaCl added to a final concentration of 0·03 M. Absolute ethanol was added at −6° and the fraction insoluble in 34% ethanol (v/v) was collected (precipitate *F*).

Isolation of amino acids

The protein samples were hydrolysed in sealed tubes for 18 hr. at 105° with 6 N-HCl, taken to dryness three times and dissolved in water. Preliminary fractionation of the amino acids was achieved in a column of Dowex 50 cation-exchange resin (200–400 mesh/in., Microchemical Specialities Co., Berkeley, California). The column (3 × 100 cm.) was packed in 1·5 N-HCl and the amino acids from 1 g. protein were added to the column in about 50 ml. water. The column was eluted with increasing concentrations of HCl as described by Stein & Moore (1950). Using this method there is incomplete separation of the amino acids, but fractionation was followed by paper chromatography of the effluents, and those fractions which contained radioactive amino acids were collected separately. [^{14}C]Serine was mixed with threonine and glutamic acid, [^{14}C]glycine with alanine, [^{14}C]valine with a trace of proline and [^{14}C]lysine with a trace of tyrosine.

Glycine. The glycine-rich fraction was allowed to react with excess fluorodinitrobenzene (FDNB) in aqueous ethanol containing $NaHCO_3$ (Sanger, 1945). The product was fractionated by the method already described (Campbell & Work, 1952) and the dinitrophenylglycine (DNP-glycine) crystallized from a mixture of *cyclo*hexane and ethyl acetate. Identity was confirmed by comparison with an authentic sample.

Serine and valine. The appropriate fraction from the Dowex column was treated as above and the DNP-amino acid purified by the appropriate method (Campbell & Work, 1952).

Lysine. The lysine-rich fraction was transferred to a column of Dowex 50 previously equilibrated with 0·2 M ammonium acetate pH 5·5 (Hirs, Moore & Stein, 1952). The tyrosine was eluted with 0·2 M ammonium acetate pH 5·5 and the lysine with 0·4 M buffer pH 5·7. Ammonium acetate was removed from the effluent by sublimation and the lysine, after conversion into DNP-lysine, was purified on silica as described by Porter (1950). The final product crystallized from a mixture of formic acid and water.

Measurement of radioactivity

Proteins. Proteins were assayed for radioactivity on 1 or 2 sq.cm. disks at infinite thickness as described previously (Campbell & Work, 1952). When counting ^{35}S, corrections were made for radioactive decay.

Glycine, serine, valine and lysine. Radioactivities were measured on suitably diluted DNP-amino acids using 1 sq.cm. disks at infinite thickness; counting was continued to an error of less than 5%. All results are expressed as counts/min./sq.cm. of pure amino acid, allowance being made for the different molecular weights of the DNP-amino acids. Each DNP-amino acid was estimated colorimetrically before dilution, as described previously (Campbell & Work, 1952), and, as a check on the accuracy of this method, larger samples of some of the crystallized DNP-amino acids were also weighed and diluted. For example, 427 and 285 μg. samples of DNP-glycine from casein measured colorimetrically and diluted as described previously gave specific activities of 10 200 and 11 700, while a larger weighed sample (4·036 mg.) gave a value of 11 400. Similarly, valine radioactivity from colorimetric estimation was 1080 and 945 and from a large sample (14·449 mg.) was 1008. All estimations were made in duplicate and in all cases duplicate values differed by less than 15%.

RESULTS

Milk composition

In earlier experiments (Campbell & Work, 1952) the assumption was made that milk composition was constant over the period of a single experiment. This assumption was checked with the goat by milking dry at 7.30 a.m., 10.40 a.m., 12.40 p.m. and 2.40 p.m. on a single day. Milk fat was removed from each sample which was then analysed for total N (Kjeldahl), non-casein N (after removal of casein at pH 4·5) and non-protein N (after removal of whey proteins with 10% trichloroacetic acid). The results given in Table 2 show that there is no

Table 2. *Effect of repeated milking on the composition of samples of goat milk*

Results are expressed as g. N/100 ml. milk.

Time of milking	Non-protein N	Non-casein N	Total N
7.30 a.m.	0·036	0·16	0·70
10.40 a.m.	0·039	0·15	0·71
12.40 p.m.	0·038	0·15	0·68
2.40 p.m.	0·036	0·16	0·70
4.40 p.m.	0·039	0·15	0·70

change in the distribution of milk nitrogen during the course of multiple milkings on a single day.

Proteins of goat milk

When examined in the electrophoresis apparatus, casein separated at pH 7·0 (sodium phosphate, $I = 0{\cdot}1$) into three components corresponding to α-, β- and γ-casein with an approximate ratio $\alpha:\beta$ of 1·8:1 and with only traces of the γ-component. The different whey-protein fractions resulting from the fractionation outlined in Table 1 were also examined in the electrophoresis apparatus using veronal buffer, pH 8·6, $I = 0{\cdot}1$. Fraction *C* was a complex mixture. Fraction *D*, the immune globulin fraction, seemed to vary in composition with different preparations. In some experiments only two components separated at pH 8·6 but in other preparations three components could be distinguished. The yield of this fraction was too small to permit further analyses.

At pH 8·6 fraction *B* showed mainly one component with an asymmetric boundary and about 10% of a faster moving component (similar to bovine lactalbumin). In cow's milk this ammonium sulphate fraction is β-lactoglobulin (Smith, 1946). Bain & Deutsch (1948) have shown that goat lactoglobulin, like bovine β-lactoglobulin (Li, 1946), forms 2 or 3 boundaries at pH values below its isoelectric point. Our goat lactoglobulin fractions were therefore examined electrophoretically in sodium acetate buffer at pH 5·1, $I = 0{\cdot}1$. In fraction *B* four boundaries separated at pH 5·1. The picture of fraction *F* (obtained by ethanol fractionation of *B*) showed a major component with a mobility of $+1{\cdot}95 \times 10^{-5}$ cm.²/v/sec. This fraction corresponded to the goat β-lactoglobulin of Bain & Deutsch (1948) obtained from milk by ethanol fractionations.

Fraction *E* (Table 1) at pH 5·1 gave a pattern closely similar to fraction *F*. The two components present had the same mobilities as those of fraction *F* and the major component represented about 85% of the total. The two fractions *E* and *F* were also compared in an analytical ultracentrifuge in sodium acetate buffer, $I = 0{\cdot}1$ at pH 5·1 (1·2% protein concentration). Both fractions formed a single homogeneous boundary with the same sedimentation constant (S_{20} about 3·0).

As an additional check on the identity of *E* and *F*, a rabbit was immunized against fraction *E* by nine intravenous injections of increasing amounts of protein (1–10 mg.) over a period of 5 weeks. Immune serum from this rabbit was put in a centre well cut in a suitable agar plate as described by Ouchterlony (1953); solutions of proteins *E* and *F* were allowed to diffuse towards the centre well from equidistant subsidiary wells and the patterns of antibody–antigen precipitation in the agar were compared. Both fractions gave similar precipitation patterns (Fig. 1). Two components in fraction *E* appeared identical with those in fraction *F* but were present in different proportions, and both fractions showed traces of a third component.

It could thus be concluded that fractions *E* and *F* contained the same two protein components. The mobility of the main component is identical with that of crystalline β-lactoglobulin (Askonas, 1954). The crystalline protein (*G*) was used for isolation of the radioactive DNP-amino acids. As a check on the reliability of the radioactivity determinations fraction *E* was also hydrolysed and used for isolation of radioactive DNP-amino acids.

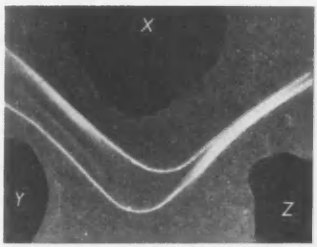


Fig. 1. Antibody–antigen precipitation pattern obtained in agar gel using method of Ouchterlony. Immune serum from a rabbit immunized against protein *E* was placed in cup *X*. A solution of protein *B* (10 mg./ml.) was put in cup *Y* and a solution of protein *E* (10 mg./ml.) in cup *Z*. The plate was covered and left at 2° for 4 weeks before taking photograph (retouched).

Non-specific adsorption of radioactivity on milk proteins

It was important to know whether non-specific adsorption of radioactive amino acids on milk proteins could be a source of serious error in estimation of radioactivity. Accordingly, 0·02 mg. of [^{35}S]methionine containing 1 μc of ^{35}S was added to 100 ml. of fresh goat milk, and after gentle stirring for 30 min. the casein and whey proteins were precipitated in the usual way. The casein contained 0·0163 μc of ^{35}S and the whey protein 0·0023 μc of ^{35}S, thus only 2% of the added [^{35}S]methionine had been precipitated with the proteins and it was clear that non-specific adsorption was not a serious source of error.

Incorporation of radioactive methionine into milk proteins of goat

The goat was given a single intravenous injection of 2 mg. [^{35}S]methionine (50 μc) and milked at frequent intervals for 8 hr. The distribution of radioactivity in casein, whey protein and blood plasma protein is shown in Fig. 2. The different proteins varied slightly in nitrogen content but the radioactivities in Fig. 2 have all been adjusted to the

same nitrogen value. Further samples of milk were obtained at 24, 32 and 48 hr. The general form of this curve is closely similar to that obtained for valine and lysine in rabbit milk (Campbell & Work, 1952) where it was found also that whey protein was slightly less radioactive than casein. The efficiency of incorporation is extremely high, about 50% of the [^{35}S]methionine appearing as protein-^{35}S within 8 hr. of injection.

Incorporation of radioactive glycine, valine and lysine into milk proteins

Owing to the varying amino acid composition of different proteins, direct comparison of radioactivities of whole proteins may be misleading. Although [^{35}S]methionine is the cheapest available radioactive amino acid, it was found to be unsuitable for more exact comparison owing to difficulty in isolation of pure methionine and to the relatively short half-life of ^{35}S. For exact comparison of the specific activities of amino acids in casein and in whey proteins we therefore used the same three amino acids as we had previously used in rabbits. In order to reduce the possibility of comparisons being invalidated by biological variability, all three amino acids were injected simultaneously immediately after milking and the animal was again milked dry 4 hr. later. The radioactive proteins were separated by the method already described (Table 1) and the distribution of radioactivity in these protein fractions was determined. As might be expected from our earlier results on the transfer of immune globulin from blood to milk

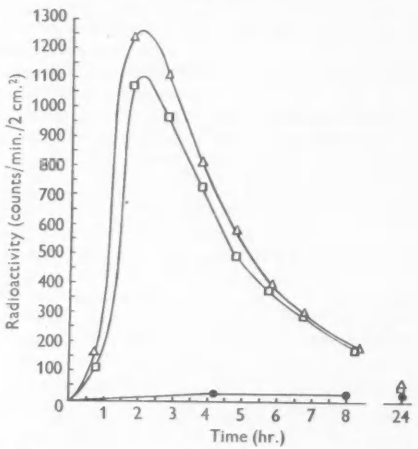


Fig. 2. The appearance of radioactivity in the milk proteins after injection of 2 mg. [^{35}S]methionine (50 μc) into a goat. △—△, casein; □—□, whey proteins; ●—●, blood plasma proteins.

Table 3. *Specific radioactivities of pure amino acids isolated from hydrolysates of milk proteins obtained 4 hr. after intravenous injection of 4·9 mg.* [^{14}C]*glycine* (250 μc), 17·3 *mg.* [^{14}C]*valine* (25 μc) *and* 75·7 *mg.* [^{14}C]*lysine* (40 μc)

	Radioactivity (counts/min./sq.cm. at infinite thickness)		
Amino acid	Casein	Fraction *E*	Cryst. lactoglobulin, *G*
Glycine	11 100	10 450	9900
Lysine	3 170	3 050	3000
Valine	1 010	1 000	950
Serine	1 510	1 040	1050

(Askonas, Campbell, Humphrey & Work, 1954) the immune globulin fraction *D* was significantly less active than the other whey fractions *B*, *C* and *E*.

Comparison of specific radioactivities of amino acids in casein and in β-lactoglobulin

Pure DNP derivatives of valine, lysine, glycine and serine were isolated from casein and from fractions *E* and *G*. The results of radioactivity determinations are shown in Table 3. In crystalline β-lactoglobulin *G*, valine, lysine and glycine had, within experimental error, the same radioactivity as in casein. The less pure β-lactoglobulin *E* was indistinguishable from crystalline β-lactoglobulin in this respect. In both proteins *E* and *G* the serine was of considerably lower specific activity than in casein.

DISCUSSION

In an earlier paper (Campbell & Work, 1952) it was shown that if two proteins are synthesized entirely from free amino acids and if the ratio between the rates of synthesis of two proteins is constant, then, after a single injection of radioactive amino acid, this amino acid would have the same specific activity in the two proteins. In our earlier experiments with rabbits it was not practicable to demonstrate the constancy of the above ratio but in the present series of experiments we have shown (Table 2) that there is no change in the ratio of casein to whey protein during the course of an experiment. We also assumed in earlier experiments that there was no selective reabsorption of milk proteins and that at the instant of injection the ratio: casein pool/whey-protein pool was proportional to the ratio: rate of casein synthesis/rate of whey-protein synthesis. The shape of the radioactivity curves for casein and for whey in Fig. 2 show that this assumption also was justified; if it were not, the two curves would cross.

In our rabbit experiments (Campbell & Work, 1952) a significant difference was found between the specific activity of amino acids isolated from casein

and from whey protein. This was taken to show that the amino acids of milk protein were obtained from two sources, the main source being free amino acids of the blood stream and the secondary source bound amino acids, either in the form of peptides or proteins. We have recently shown that the immune globulin of milk does not equilibrate with injected radioactive amino acid and that some of the bound amino acid transferred from blood to milk is thus accounted for as immune globulin (Askonas, Campbell, Humphrey & Work, 1954). It seemed possible that other blood proteins might be transferred to the milk in the same way and that this could account entirely for the differences in specific radioactivity between casein and whey protein. From the present comparison between the radioactivity of amino acids isolated from casein and β-lactoglobulin (Table 3) (neglecting for the moment the serine value which was obtained after injection of [^{14}C]glycine and not of serine) it would seem that the nitrogen pool for the synthesis of casein and of lactoglobulin is identical and that this pool is in equilibrium with free circulating amino acids. According to this result it is unlikely that either protein derives much nitrogen from blood peptides or from partial hydrolysis and rearrangement of blood proteins. Our results are, however, not conclusive on this point. We could suppose that bound amino acid in the form of peptide was used to an equal extent in the synthesis of casein and β-lactoglobulin. This assumption is improbable, partly because of the very different amino acid composition of casein and whey proteins and partly because we have been unable to find evidence for the incorporation of bound amino acids into casein after partial hydrolysis and isolation of the amino acids from different peptide sequences thus obtained (Askonas, Campbell & Work, 1954).

One point in the present results requires further comment, the low value of the specific radioactivity of serine in proteins *E* and *G* (Table 3). Serine was not one of the injected radioactive amino acids but was produced very rapidly from injected [^{14}C]-glycine. Since no significant difference was found between the casein and the β-lactoglobulin for the [^{14}C]amino acids injected, the difference between these and serine might be attributed to the nature of the process involved in the conversion of glycine into serine *in vivo*. It may be suggestive that the serine of casein is present partly as serine and partly as serine phosphate.

SUMMARY

1. A lactating goat was injected with radioactive glycine, valine, lysine and methionine and the appearance of radioactivity in the milk proteins was followed.

2. After injection of [^{35}S]methionine, both casein and whey proteins reached maximum activity in about 1·5 hr. The whey activity curve followed closely the casein curve but was always slightly below it.

3. After simultaneous injection of [2-^{14}C]glycine, DL-[4-^{14}C]valine and DL-[1-^{14}C]lysine, milk whey was fractionated and a crystalline β-lactoglobulin was isolated. This protein and a sample of casein from the same milk were hydrolysed and the radioactive dinitrophenyl amino acids isolated in a pure state.

4. There was no significant difference in radioactivity between glycine, valine and lysine from casein and the corresponding amino acids from lactoglobulin. Casein serine was more active than lactoglobulin serine. The significance of these results is discussed.

We wish to thank Mr J. Coote and Miss M. Smith for their careful assistance and Mrs A. Dovey and Dr J. Humphrey for valuable advice.

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Crystallization of Goat β-Lactoglobulin

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Bain & Deutsch (1948) obtained lactoglobulin from goat milk by carrying out two ethanol fractionations of the whey proteins. The fraction they referred to as 'goat lactoglobulin' gave a single peak on electrophoresis at pH 8·0 in barbiturate buffer, but three boundaries separated at pH 7·0 and at least two at pH 4·2–6·0 after 2 hr. at 4·5 v/cm., $I = 0{\cdot}1$. The two main components had their isoelectric points at pH 5·2 and 5·9 respectively in sodium acetate buffer, $I = 0{\cdot}1$. 'Goat lactoglobulin' thus behaved in much the same way as crystalline bovine β-lactoglobulin, which also gives a single peak on electrophoresis at pH 8 but forms two or more boundaries at pH's 4·8 and 6·2 (Li, 1946). According to Polis, Schmukler, Custer & McMeekin (1950), crystalline bovine β-lactoglobulin shows multiple peaks at pH values acid to its isoelectric point but below pH 3·0 becomes once more a single peak.

In the present study goat lactoglobulin was crystallized from ammonium sulphate, and its electrophoretic behaviour was investigated at pH values ranging from pH 4·1 to 8·0. The three- and four-times recrystallized material appeared to be homogeneous on electrophoresis at pH 4·1 and 5·6–8·6, forming single symmetrical boundaries in runs lasting up to seven hours at about 5 v/cm. However, fairly concentrated solutions (above 1 %), analysed at pH 4·6 and 5·1 in sodium acetate buffer, $I = 0{\cdot}1$, produced markedly asymmetrical boundaries in the descending limb. The isoelectric point, sedimentation constant and molecular weight of the crystalline goat β-lactoglobulin were determined and some of its properties compared with those of crystalline bovine β-lactoglobulin.

METHODS

Goat milk was partially defatted by centrifuging at about 1500 *g*. The casein was precipitated by the dropwise addition of a mixture of N-HCl and N acetic acid (1:2, v/v) until the pH reached 4·5 and the casein precipitate was centrifuged off.

Preliminary fractionation of whey with $(NH_4)_2SO_4$

The pH of the whey was adjusted to 5·8 with 0·1 N-NaOH and solid $(NH_4)_2SO_4$ was added slowly with constant stirring to a saturation of 0·58 (380 g./l.). After standing for at least 1 hr. the precipitate was filtered off on a pad of Celite 545 (Johns Manville Co., London), previously washed with a solution of $(NH_4)_2SO_4$ (0·58 saturated). The precipitate was discarded. Solid $(NH_4)_2SO_4$ was then added to the clear supernatant to 0·9 saturation (requiring 230 g./l.) and the mixture left to stand several hours or overnight at room temperature. The mixture was filtered on a pad of Celite 545 and the filtrate discarded. The fraction insoluble in 0·58–0·9 saturated $(NH_4)_2SO_4$ was washed off the Celite with a minimum volume of 5 % $(NH_4)_2SO_4$, dialysed for several hours against running tap water and then 48 hr. against four changes of distilled water. The dialysed material was freeze-dried *in vacuo* and contained β-lactoglobulin.

Crystallization of goat β-lactoglobulin

$(NH_4)_2SO_4$ (A.R.) was used and all steps were carried out at room temperature except for high-speed centrifuging at 4·5°. The above freeze-dried protein fraction (4–6 g.) was dissolved in 100 ml. water and the pH of the solution adjusted to pH 6·5. An equal volume of saturated $(NH_4)_2SO_4$ was added slowly with stirring and after 20 min. the small amount of precipitate was removed by filtration through Celite 545, previously washed with the same $(NH_4)_2SO_4$ mixture. The precipitate was discarded. Solid $(NH_4)_2SO_4$ (65 g./l. of filtrate) was then added slowly. After 1 hr. the precipitate (insoluble in 0·58–0·67 saturated $(NH_4)_2SO_4$) was spun down at 4·5° in a refrigerated high-speed centrifuge at about 15000 *g*. A further 65 g. $(NH_4)_2SO_4$/l. of supernatant precipitated the protein insoluble at 0·67–0·76-saturated $(NH_4)_2SO_4$ and the precipitate was spun down in the high-speed centrifuge. A third fraction insoluble at 0·76–0·86 saturation was precipitated by the addition of a further 70 g. $(NH_4)_2SO_4$/l.

Each of the three protein fractions thus obtained was treated as described below. A small amount of water was added slowly to each fraction until most of the protein had dissolved. The amorphous precipitates were centrifuged down at about 15000 *g* and the clear supernatant left to crystallize at room temperature in a desiccator over P_2O_5. With the right conditions crystals formed overnight. For this step high concentrations of protein (5–10 %) are necessary.

Recrystallization. The crystal crops were collected by centrifuging and all samples recrystallized 3–4 times for the electrophoretic study. The crystals were washed with 0·62 saturated $(NH_4)_2SO_4$, dissolved in 0·5 saturated $(NH_4)_2SO_4$ and several drops of water if necessary. Solid $(NH_4)_2SO_4$ was added to cloudiness, and the samples were left to crystallize as before. Recrystallization of purified material may be carried out from more dilute solutions (about 1 % of protein) and if the protein is allowed to crystallize slowly over several days, perfectly shaped crystals up to 1–2 mm. in size may be obtained. Since the protein is more insoluble at high temperature it is important to avoid sudden increases in temperature after crystallization to prevent formation of

amorphous precipitates. The yield varied from 10 to 25% of the first $(NH_4)_2SO_4$ precipitate (0·58–0·9-saturated $(NH_4)_2SO_4$) depending on the scale of the experiment and the age and purity of the crude fraction.

Electrophoresis

Electrophoretic analyses were carried out in a Hilger Tiselius apparatus at +1° using acetate, phosphate or veronal buffers ($I = 0·1$), at pH 4·1–8·0, with a potential gradient of about 5 v/cm. The 3–4 times recrystallized material was dissolved to yield solutions containing 0·5–1·3% of protein. The protein concentration could be estimated by measuring the refraction of the solution, or its ultraviolet absorption at 278 mμ. (the absorption maximum) in a Unicam spectrophotometer. In each case the descending boundary was utilized for the calculation of the electrophoretic mobility.

Ultracentrifuge experiments

Runs were carried out in a Spinco ultracentrifuge, analytical model E, at 187 100 or 259 700 *g*. The temperature of the free rotor couple was read at the start and finish of each run. Each point was corrected for temperature assuming an even change over the period of the run. The instrument has not been calibrated for actual cell temperature; hence the values were not corrected for the error arising from any difference between free couple and true cell temperature. Protein concentration was determined as described for the electrophoretic analyses. Solutions of protein (0·2–1%) were dialysed for 48 hr. against phosphate buffer pH 6·9, $I = 0·2$, containing 0·1 M-NaCl, 0·0188 M-KH_2PO_4 and 0·0271 M-Na_2HPO_4.

S_{20} was calculated by the method described by Cecil & Ogston (1948); 0·751 being taken as the value for the partial specific volume of the protein. This is the value given by Pedersen (1936) for bovine β-lactoglobulin.

RESULTS

Crystalline form

β-Lactoglobulin from goat milk crystallizes from approximately 0·6–0·65 saturated ammonium sulphate solution in well-formed hexagonal bipyramids (Fig. 1). The actual ammonium sulphate concentration required depends on the concentration of protein present.

The solubility of the goat lactoglobulin crystals in ammonium sulphate is very similar to that of the needles obtained by Sørensen & Sørensen (1939) from cow's milk by similar methods. There are no electrophoretic data on the crystalline material obtained by Sørensen & Sørensen, and crystallization appears to have been very slow, taking at least 8–14 days, whereas in the present study the goat lactoglobulin forms crystals overnight in ammonium sulphate under the right conditions.

Crystalline bovine β-lactoglobulin is prepared usually by dialysing the protein at its isoelectric point (pH 5·2) free from salt (Palmer, 1934). Attempts at crystallization of the goat lactoglobulin by dialysing the preparation free from salt at its isoelectric point (pH 5·9) at 0° for 1–2 weeks have been unsuccessful so far, although as with bovine lactoglobulin (Palmer, 1934) an oily layer separated after dialysis against de-ionized water at pH 5·9.

A second crystalline form was at times obtained from the mother liquor after the hexagonal bipyramids had crystallized out. These crystals were highly birefringent and cubic in shape, but they were obtained in amounts too small for extensive investigation. They had the same sedimentation constant as the other type of crystalline protein ($S_{20} = 2·87$ in phosphate–NaCl buffer pH 6·9, $I = 0·2$, at a concentration of 0·52% protein). The electrophoretic mobility determined in three runs at different pH values (0·7–0·8% protein) was slightly higher (around 10%) than that of the usual type of crystalline form of goat β-lactoglobulin (see Fig. 2 for mobility of the hexagonal crystals at different pH values). The crystals shaped like cubes had the following electrophoretic mobilities (in cm.²/v/sec.) at an ionic strength of 0·1: $+3·8 \times 10^{-5}$ and $+2·1 \times 10^{-5}$ in sodium acetate buffer at pH 4·55 and 5·1 respectively, and $-2·4 \times 10^{-5}$ in phosphate buffer at pH 7·0. It is, therefore, not quite clear whether these are a different form of β-lactoglobulin crystals or crystals of a different protein. All other results in this study refer to the goat β-lactoglobulin crystallized as shown in Fig. 1.

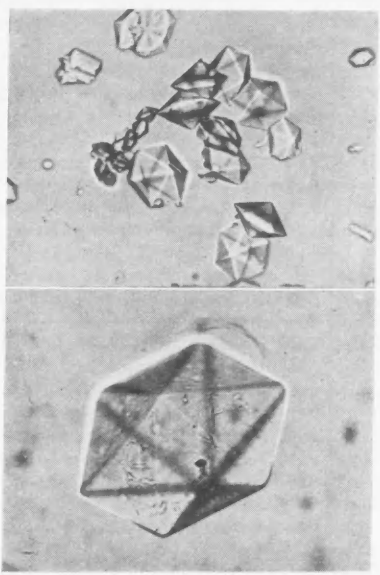

Fig. 1. Goat β-lactoglobulin crystallized from $(NH_4)_2SO_4$. Magnification, large crystal × 270; small crystals × 156.

Electrophoretic behaviour

Isoelectric point. In Fig. 2, mobility of the crystalline goat β-lactoglobulin is plotted against the pH of the electrophoretic runs. It may be seen that in sodium acetate, $I = 0{\cdot}1$, the isoionic point of the protein lies at pH 5·9. Goat lactoglobulin thus has a higher isoelectric point than bovine lactoglobulin (pH 5·2 in the same buffer, cf. Longsworth & Jacobsen, 1949). The value obtained agrees with that quoted by Bain & Deutsch (1948) for one of the components of their goat lactoglobulin. It may also be noted on Fig. 2 that increasing the ionic strength of the sodium acetate buffer to 0·2 at pH 4·6 reduces the mobility of the protein from 3·4 to $2{\cdot}3 \times 10^{-5}$. The same effect was noted by Longsworth & Jacobsen (1949) with bovine β-lactoglobulin and with serum albumin, suggesting binding of the salt by the protein.

Homogeneity of crystalline goat β-lactoglobulin. Fig. 3 shows some of the electrophoretic patterns obtained by examining 3- to 4-times recrystallized lactoglobulin at different pH values. The material appears to be homogeneous, forming a perfectly symmetrical boundary at pH 5·6–8·0 and at pH 4·15 using 0·7–1·5 % solution of protein (see Fig. 3, *a*, *b*, *c*, *g*). If electrophoretic analysis is carried out at pH 5·1 and about 4·6 in acetate buffer, $I = 0{\cdot}1$, using solutions of protein at concentrations higher than 1 %, the descending boundary is markedly asymmetric in runs lasting for 4–6 hr. (Fig. 3, *d*, *e*).

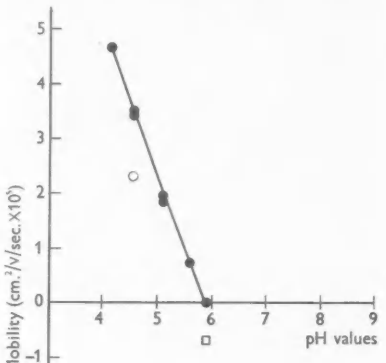

Fig. 2. Electrophoretic mobilities of crystalline goat β-lactoglobulin at pH 4·1–8·1. ●, Sodium acetate buffer, $I = 0{\cdot}1$; ○, sodium acetate buffer, $I = 0{\cdot}2$; □, sodium phosphate buffer, $I = 0{\cdot}1$; ×, veronal buffer, $I = 0{\cdot}1$.

At pH values below the isoelectric point of the protein there is a striking difference between the patterns of the ascending and descending boundaries. The ascending boundary stays extremely sharp throughout the whole run with no evidence of any asymmetry or of a second component. This same sharpening effect has been described by Longsworth & Jacobsen (1949) for electrophoresis of bovine β-lactoglobulin and serum albumin. When the ionic strength of the acetate buffer was raised to 0·2, a 1·2 % solution of protein produced a symmetrical peak even after a 7 hr. run (Fig. 3, *f*).

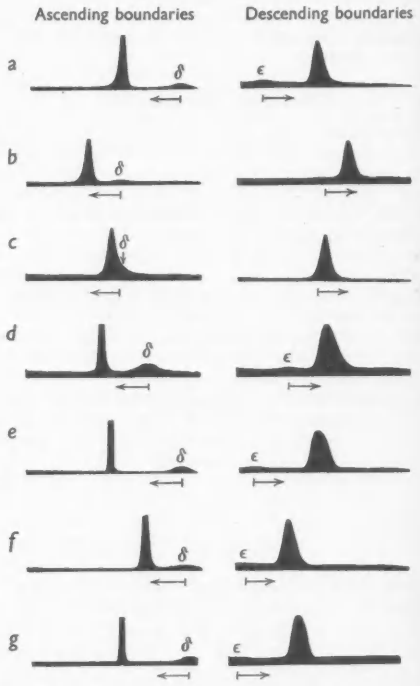

Fig. 3. Electrophoretic patterns of crystalline goat β-lactoglobulin. The arrows below the patterns indicate the position of the boundary at start of run and direction of movement. Potential gradient was about 5 v/cm. $I = 0{\cdot}1$, unless otherwise indicated.

	Buffer	pH	Protein concentration (%)	Duration of run (hr.)
a	Sodium veronal	8·1	1	4·5
b	Sodium phosphate	7·05	1	4·0
c	Sodium acetate	5·9	1·3	6·5
d	Sodium acetate	5·1	1·2	5·25
e	Sodium acetate	4·55	1·3	4·25
f	Sodium acetate ($I = 0{\cdot}2$)	4·65	1·2	7·0
g	Sodium acetate	4·15	1	3·5

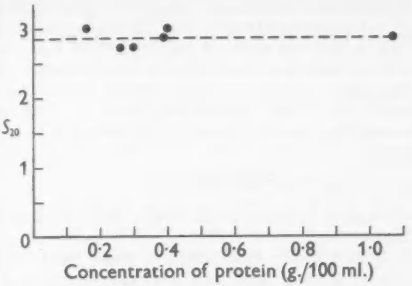

Fig. 4. Sedimentation constant of crystalline goat β-lactoglobulin at different protein concentrations. Average $S_{20} = 2{\cdot}85$.

Sedimentation constant

Fig. 4 shows several values of S_{20} obtained by using different concentrations of crystalline goat β-lactoglobulin. Ultracentrifuging was carried out at pH 6·9 in phosphate–sodium chloride buffer, $I = 0{\cdot}2$. It is not possible to extrapolate these few values to infinite dilution. They suggest that S_{20} is independent of the protein concentration. More points would be required to establish whether the apparent dip in S_{20} between 0·2 and 0·4 % protein is significant or whether the spread in the points is due purely to experimental error. The six values were, therefore, averaged, giving a mean value of 2·85 for S_{20} at pH 6·9.

DISCUSSION

Comparison with bovine β-lactoglobulin

The goat β-lactoglobulin appears to be a molecule similar in size to that of bovine β-lactoglobulin. D_{20} and S_{20} are of the same order in the two proteins. A number of values for S_{20} of bovine lactoglobulin have been reported in the literature. Thus Pedersen (1936) obtained mean values of 3·12 at pH 5·2–5·7. Cecil & Ogston (1949) observed an S_{20} of 2·83 at infinite dilution in sodium acetate buffer, pH 5·14, and Miller & Golden (1952) a value of 2·91 at pH 5·0 using a Spinco ultracentrifuge. The authors found the usual decrease in S_{20} with increasing protein concentration.

The sedimentation constant of crystalline goat β-lactoglobulin was found to equal 2·85 (mean value, 0·2–1 % protein) at pH 6·9 in phosphate buffer, $I = 0{\cdot}2$. S_{20} appeared independent of the protein concentration but is very similar to the values reported for the bovine β-lactoglobulin.

The diffusion coefficients were determined on solutions containing 0·26 and 0·38 % protein (see Addendum). It may be assumed that D_{20} shows no appreciable variation at this range of concentration (cf. Cecil & Ogston, 1949); therefore the two values of D_{20} obtained were averaged. Thus D_{20} of 7·48 and S_{20} of 2·85 gave a molecular weight of 37100 for goat β-lactoglobulin. This lies within the range of values (35000–39000) reported by various authors for the molecular weight of bovine lactoglobulin (cf. Cecil & Ogston, 1949; Pedersen, 1936; Polis *et al.* 1950; Bull & Currie, 1946).

Bovine β-lactoglobulin has been the subject of extensive study. For a long time it was believed to be a good example of a homogeneous protein. Li (1946), however, showed that although the crystalline bovine β-lactoglobulin appeared to be homogeneous at pH 7 and 8·6, electrophoresis at pH 4·8 and 6·2 showed a heterogeneous pattern with two to three components separating. This heterogeneity has been investigated by McMeekin, Polis, Della-Monica & Custer (1948) and Polis *et al.* (1950). Electrophoresis of 4-times recrystallized bovine β-lactoglobulin in sodium acetate buffer pH 4·8 showed 40 % of a slow- and 60 % of a fast-moving component. Although the ascending and descending boundaries were not mirror images, both showed two components. Polis *et al.* (1950) isolated the slower moving component (β_1) in crystalline form in small yield (1 g. from 50 g. crystalline β-lactoglobulin). This component had the same molecular weight as β-lactoglobulin and the suggestion of Lundgren & Ward (1949) that the heterogeneity is due to the presence of immune globulin is thus ruled out.

That the different components, if they are different proteins, interact strongly has been shown by Polis *et al.* (1950). Substitution of sodium chloride for sodium acetate at a constant ionic strength of 0·1 changes the proportion of the two components in electrophoretic patterns of bovine β-lactoglobulin.

Heterogeneous electrophoretic patterns have also been noted with other proteins previously presumed to be pure, when electrophoresis is carried out in sodium acetate buffer at pH values below the isoelectric point. Li & Pedersen (1953) found aggregation of growth hormone molecules at pH 4·0 in sodium acetate buffer with a preparation homogeneous at pH 2·32 and 9·93. In crystalline human and horse serum albumins, also, more than one component may be identified by electrophoresis at pH 4·0 in sodium acetate buffer, $I = 0{\cdot}02$ (Luetscher, 1939).

The present study showed that crystalline goat lactoglobulin formed symmetrical boundaries at pH 4·1 and pH 5·6–8·0. Electrophoresis continued for more than 4 hr. at pH 4·6 and 5·1 in sodium acetate, $I = 0{\cdot}1$, at a protein concentration over 1 %, however, produced asymmetric boundaries in the descending limb. Under the conditions of electrophoresis used it has not been possible to get separation of more than one component. It is not quite

clear whether the asymmetry of the descending boundary is due to the presence of more than one protein component in the crystal or merely to some reversible change in the molecule below the isoelectric point of the protein. It is not likely that the asymmetry is due to changes in the size of the molecule. Ultracentrifuging of a 0·5 % solution for 2 hr. at 187 100 *g* in sodium acetate (pH 4·6, $I = 0·2$) showed no sign of heterogeneity. Any striking change in the size of the molecule by dissociation or aggregation at pH 4·6 should show up by this method.

SUMMARY

1. β-Lactoglobulin may be crystallized from goat milk by means of ammonium sulphate. The isoelectric point of the protein lies at pH 5·9 in sodium acetate buffer, $I = 0·1$.

2. The electrophoretic behaviour of the protein was studied at pH values ranging from 4·1 to 8·1. The protein appears homogeneous forming a symmetrical boundary at pH 4·15 and 5·6–8·1. An asymmetric boundary may be noted in the descending limb at about pH 4·6 and 5·1 in sodium acetate buffer, $I = 0·1$. This asymmetry disappears on raising the concentration of the sodium acetate to an ionic strength of 0·2 at pH 4·65.

3. The goat β-lactoglobulin was found to have a molecular weight of the same order as bovine β-lactoglobulin (i.e. 37 100). S_{20} was 2·85 and D_{20} $7·48 \times 10^{-7}$ (see Addendum).

I should like to express my thanks to Dr T. S. Work and Dr P. N. Campbell for their interest and encouragement and to Dr R. A. Kekwick and Mr E. Caspary for their helpfulness with the sedimentation and diffusion measurements. I am most grateful to Mrs A. Dovey for carrying out the electrophoretic runs, to Mr J. Orr for doing the runs in the Spinco ultracentrifuge and Mr Young for the photographs of the crystals.

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ADDENDUM

Diffusion Coefficient of Crystalline Goat β-Lactoglobulin

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The diffusion measurements were carried out by the Gouy interferometric method (Kegeles & Gosting, 1947) in a modification of the apparatus of Gosting, Hanson, Kegeles & Morris (1949) with a parallel light beam through the cell at 25 ± 0·01°. Increased sensitivity was obtained by using the blue line, $\lambda = 4360$ Å, isolated from a high-pressure mercury arc by an interference filter. Boundaries were made by flowing through a capillary (Kahn & Polson, 1947).

Diffusion coefficients were measured in the same phosphate–sodium chloride buffer used for the sedimentation-runs (pH 6·9, $I = 0·2$) at two concentrations of protein. $D_{20,w}$ was found to equal 7·38 and $7·58 \times 10^{-7}$ cm.²/sec. using 0·38 and 0·26 % solutions of protein respectively. Only slight deviation from the ideal form, defined by the probability integral, was observed.

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Stimulation of Respiration by 2:4-Dinitrophenol

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It has been known for some time that 2:4-dinitrophenol (and other nitrophenols) stimulate the respiration of intact cells, while at the same time the energy-requiring functions of the cell are impaired.

The nature of the effect on the energy-requiring functions became clearer with the discovery by Loomis & Lipmann (1949), and Cross, Taggart, Covo & Green (1949) that dinitrophenol 'uncoupled' oxidative phosphorylation, i.e. it inhibited the synthesis of adenosine triphosphate (ATP) without inhibiting respiration. However, the mechanism of the stimulation of respiration is still obscure.

An important advance towards the elucidation of this mechanism was made by Potter & Recknagel (1951) and Lardy & Wellman (1952, 1953), who defined the conditions for demonstrating the stimulation of the respiration of isolated liver mitochondria by dinitrophenol. Carefully prepared liver mitochondria with low ATPase activity (Kielley & Kielley, 1951) respire only slowly in the absence of a phosphate-acceptor system. The respiration is greatly stimulated by adding either a phosphate-acceptor system or dinitrophenol.

Lardy's (1952) results show that these two effects are not additive, i.e. the addition of dinitrophenol to mitochondria already supplied with hexokinase and glucose has little effect on the respiration. The present paper shows that heart sarcosomes (mitochondria) behave in the same way. However, as already reported in a short communication (Slater & Lewis, 1953), the rate of oxidation of α-ketoglutarate (α-oxoglutarate) by sarcosomes isolated from the thoracic muscle of the blowfly (*Calliphora erythrocephala*) is markedly increased by 2:4-dinitrophenol, even in the presence of hexokinase and glucose. Terner (1953) has found that homogenates of the mammary gland behave similarly. The present paper elaborates and extends our previous results.

METHODS

Heart sarcosomes. These were prepared from rat heart, as described by Cleland & Slater (1953), using as isolation medium a solution of 0·32 M sucrose and 0·01 M ethylenediaminetetraacetate, adjusted to pH 7·4.

Blowfly sarcosomes. These were prepared as described by Lewis & Slater (1954).

Activity of α-ketoglutaric oxidase system. The activities are expressed as Q_{O_2}, calculated from the α-ketoglutarate (αKg) disappearance as described by Lewis & Slater (1954).

P:O *and* P:αKg *ratios.* These were measured as described in previous papers (Slater, 1953*a*; Slater & Holton, 1953).

RESULTS

α-Ketoglutaric Oxidase System

Effect of dinitrophenol on rate of oxidation of α-ketoglutarate

The effect of various concentrations of dinitrophenol on the rate of oxidation of α-ketoglutarate by different sarcosomal preparations is shown in Fig. 1. Results with a larger number of preparations at a single concentration of dinitrophenol (10^{-4} M) are summarized in Table 1. In all cases, a large excess of hexokinase, together with glucose, was present in the reaction mixture.

The rate of oxidation of α-ketoglutarate by rat-heart sarcosomes was little affected by dinitrophenol. The behaviour of blowfly sarcosomes was variable, in some cases a large stimulation was observed, in other cases there was little action. The large effect was obtained only with sarcosomes made from young blowflies in media containing ethylenediaminetetraacetate (Expts. 3, 4, 6). A smaller activation was obtained with one preparation (Expt. 8), also made from young flies, isolated with sucrose. Another preparation from young flies isolated with the same medium was not activated. The effect of age of the blowflies is most clearly shown in Table 1 by comparing Expts. 6 and 5, with sucrose–ethylenediaminetetraacetate medium, since these preparations were made from the same batch of flies, at a 7-day interval. The effect of including ethylenediaminetetraacetate in the medium is best shown by comparing the two Expts. 6 in Table 1.

The influence of the age of blowfly on the effect of dinitrophenol has not yet been thoroughly studied. Whether it is an effect of age *per se* or a reflexion of some other condition (e.g. activity) cannot be stated.

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Table 1. *Effect of 2:4-dinitrophenol on rat-heart and blowfly sarcosomes*

The figures in brackets after 'blowfly thorax' are the ages (in days) of the blowflies. Concn. of dinitrophenol, 10^{-4} M.

Expt.	Tissue	Isolation medium	Q_{O_2} In absence of dinitrophenol	Q_{O_2} In presence of dinitrophenol	Activation (+) or inhibition (−) (%)	P:O in absence of dinitrophenol	P:αKg in presence of dinitrophenol
1	Rat heart	0·28 M Sucrose +0·01 M ethylenediaminetetraacetate, pH 7·4	59	62	+5	2·75	0·74
2	Rat heart		71	60	−16	2·72	0·64
3	Blowfly thorax (1)	0·20 M Sucrose +0·002 M ethylenediaminetetraacetate, pH 7·4	24	52	+118	0·95	0·91
4	Blowfly thorax (2)		16	55	+251	1·03	0·83
5	Blowfly thorax (9)		9·2	8·8	−3	1·68	0·71
6	Blowfly thorax (2)	0·32 M Sucrose +0·01 M ethylenediaminetetraacetate, pH 7·4	11·2	19·1	+71	0·88	0·74
7	Blowfly thorax (2)		15·9	23·2†	+46	1·61	0·66
5	Blowfly thorax (9)		8·4	8·8	+5	1·79	0·69
6	Blowfly thorax (2)	0·36 M Sucrose +0·005 M phosphate, pH 7·4	20·1	19·4	−3	1·03	0·70
8	Blowfly thorax (1–7)		19·1	27·0‡	+39	0·57	0·58
9	Blowfly thorax (6–13)		11·3	10·3	−9	0·72	0·39
10	Blowfly thorax (8–15)		11·6	13·0	+12	0·94	0·88
10	Blowfly thorax (8–15)*		8·5	7·1	−16	0·58	0·32
9	Blowfly thorax (6–13)	0·16 M Phosphate, pH 7·0 +2·5% albumin	46	46	0	0·80	0·79

* Thoraces ground in mortar for 1 min. with medium (see p. 209 of Lewis & Slater, 1954).
† 43, in presence of 5×10^{-4} M dinitrophenol. ‡ 42, in presence of 10^{-3} M dinitrophenol.

Fig. 1. Effect of 2:4-dinitrophenol on the rate of oxidation of α-ketoglutarate by rat-heart sarcosomes and by various preparations of blowfly thoracic muscle sarcosomes. ●—●, Rat-heart sarcosomes isolated in sucrose + ethylenediaminetetraacetate. ○—○, blowfly sarcosomes isolated in sucrose. +—+, blowfly sarcosomes isolated in sucrose + ethylenediaminetetraacetate. The numbers against each curve refer to the Expt. nos. in Table 1.

Fig. 2. Effect of 2:4-dinitrophenol on P:αKg ratios. The symbols and numbers are the same as in Fig. 1.

Effect of dinitrophenol on phosphorylation ratios

The effect of varying concentrations of dinitrophenol on the phosphorylation ratios of the same five preparations illustrated in Fig. 1 is shown in Fig. 2. P:αKg is plotted rather than P:O because phosphorylation associated with oxidation beyond

succinate is completely uncoupled by dinitrophenol. The following points are noteworthy. (1) Phosphorylation is largely uncoupled by low concentrations of dinitrophenol, the rat-heart sarcosomes requiring higher concentrations than the blowfly sarcosomes. (2) There is a considerable dinitrophenol-resistant phosphorylation, P:αKg remaining almost constant between about 5×10^{-5} M (10^{-4} M in the case of the heart sarcosomes) and 5×10^{-4} M dinitrophenol.

One of the experiments (Expt. 4) included in Figs. 1 and 2 is shown in greater detail in Fig. 3. It should be noted that unlike Figs. 1 and 2, the concentration scale in Fig. 3 is linear, not logarithmic. The most interesting point about Fig. 3 is the effect of different concentrations of dinitrophenol on the esterification of phosphate. Low concentrations of dinitrophenol (10^{-5} M) sharply decrease the amount of esterified phosphate, but further additions increase it again, until with 10^{-4} M dinitrophenol the esterification of phosphate is actually 36 % greater than in the absence of dinitrophenol. In another experiment (Expt. 3 of Table 1), this concentration of dinitrophenol increased the amount of esterified phosphate by 22 %.

Effect of albumin

It has been shown in the previous paper (Lewis & Slater, 1954; cf. Sacktor, 1954) that the addition of low concentrations of bovine-plasma albumin to the reaction mixture markedly accelerates the rate of oxidation of α-ketoglutarate, with little or no effect on the phosphorylation ratios. It was obviously of interest to determine the effect of dinitrophenol in the presence of albumin. Four experiments are shown in Table 2, one with a preparation giving a stimulation of the rate of oxidation of

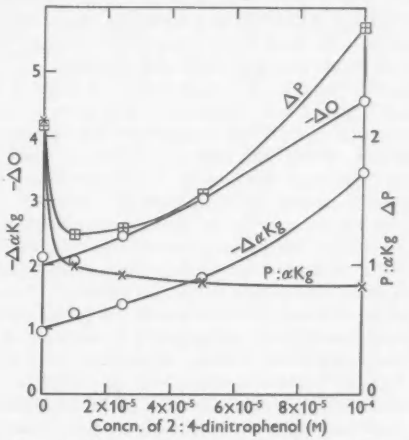

Fig. 3. Expt. 4 of Figs. 1 and 2 and Table 1 shown in greater detail. Note that the concentration of dinitrophenol is plotted on a linear scale, not logarithmic as in Figs. 1 and 2.

Table 2. *Effect of dinitrophenol in the presence and absence of albumin*

Sarcosomes isolated in 0·20 M sucrose + 0·002 M ethylenediaminetetraacetate, pH 7·4, in Expt. 1; in 0·32 M sucrose + 0·01 M ethylenediaminetetraacetate, pH 7·4, in Expts. 2–4. The ages of the blowflies were: Expt. 1, 9 days; Expt. 2, 9 days (same batch as in Expt. 1); Expt. 3, 2 days; Expt. 4, 8 days.

Expt.	Albumin (mg./ml.)	Dinitrophenol (M)	−ΔO	−ΔαKg	ΔO:ΔαKg	P:O	P:αKg
1	0	0	1·73	0·73	2·37	1·68	4·00
	1·2	0	2·64	1·26	2·10	1·72	3·61
	0	10^{-4}	1·42	0·71	2·01	0·35	0·71
	1·2	10^{-4}	1·99	0·96	2·07	0·46	0·96
2	0	0	1·65	0·61	2·70	1·79	4·81
	1·2	0	2·66	1·20	2·22	1·79	3·97
	0	10^{-4}	1·35	0·64	2·10	0·33	0·69
	1·2	10^{-4}	1·99	0·94	2·11	0·44	0·94
3	0	0	1·43	0·69	2·08	1·55	3·22
	1·2	0	2·32	1·19	1·95	1·87	3·65
	0	10^{-4}	1·59	1·01	1·58	0·42	0·66
	1·2	10^{-4}	2·20	1·44	1·53	0·55	0·84
	0	2×10^{-4}	1·77	1·45	1·22	0·49	0·59
	1·2	2×10^{-4}	1·99	1·43	1·39	0·52	0·71
	0	5×10^{-4}	2·16	1·81	1·19	0·49	0·59
4	0	0	0·89	0·53	1·68	1·46	2·45
	1·2	0	1·60	0·90	1·78	1·78	3·16
	5·0	0	1·80	0·98	1·84	1·91	3·51
	10·0	0	2·06	0·89	2·31	1·99	4·60
	0	2×10^{-4}	0·74	0·33	2·26	0·39	0·88
	1·2	2×10^{-4}	0·94	0·46	2·06	0·39	0·81
	5·0	2×10^{-4}	1·51	0·75	2·01	0·35	0·71
	10·0	2×10^{-4}	1·80	0·79	2·28	0·39	0·90

α-ketoglutarate on addition of dinitrophenol (Expt. 3) and the others with preparations not showing this stimulation.

In the absence of dinitrophenol, albumin stimulated the oxidation of α-ketoglutarate to about the same degree with all four preparations (73, 97, 73 and 70 % stimulation, respectively, in Expts. 1–4 by 1·2 mg. albumin/ml.), although they differed markedly in their response to dinitrophenol. This shows that albumin and dinitrophenol do not stimulate by the same mechanism. In Expt. 3, the stimulation of α-ketoglutarate utilization by dinitrophenol (5×10^{-4} M) exceeded that given by albumin, which was used in an amount found to give maximum stimulation in the previous paper (Lewis & Slater, 1954). Although these two activators appear then to act by different mechanisms, their effects were not additive. In fact, dinitrophenol markedly depressed the activating effect of albumin, even when it had no effect on its own (Expts. 1, 2 and 4). In Expt. 3, 2×10^{-4} M dinitrophenol completely prevented the activation by 1·2 mg. albumin. Expt. 4 shows that larger amounts of albumin (5 and 10 mg.) were able to overcome this depressing effect of dinitrophenol.

Another effect of albumin is also shown in Table 2; the P:αKg ratio with 10^{-4} M dinitrophenol is appreciably higher in the presence of albumin, approaching a value of 1 in Expts. 1 and 2.

Effect of concentration of α-ketoglutarate

It has been shown in the previous paper (Lewis & Slater, 1954) that the rate of oxidation of α-ketoglutarate by blowfly sarcosomes is markedly affected by increasing the concentration of α-ketoglutarate between 0·002 and 0·01 M, suggesting a possible permeability barrier to α-ketoglutarate. It was important, therefore, to determine if dinitrophenol promoted the oxidation of α-ketoglutarate by increasing the rate of penetration of the substrate to the α-ketoglutaric dehydrogenase. This was tested by measuring the effect of dinitrophenol at different concentrations of α-ketoglutarate. The results, summarized in Table 3, show that the effect of dinitrophenol increases with increasing concentration of α-ketoglutarate. This is contrary to what would be expected if dinitrophenol were affecting the accessibility of α-ketoglutarate to its dehydrogenase, but suggests rather that dinitrophenol activates the α-ketoglutaric oxidase system at some point other than the reaction between α-ketoglutarate and the dehydrogenase.

Succinic Oxidase System

Table 4 shows the effect of dinitrophenol on the oxidation of succinate and on the P:O ratio. In Expt. 1, the sarcosomal preparation was one which showed a marked stimulation of the α-ketoglutaric oxidase system by adding dinitrophenol; similarly, the rate of oxidation of succinate was considerably increased (by 66 %). In Expt. 2, on the other hand, in which sarcosomes from older blowflies were used, dinitrophenol had no effect on the rate of oxidation of either α-ketoglutarate or succinate. Similarly, it had no appreciable effect on the rate of oxidation of succinate by heart sarcosomes (see Table 7 of Slater & Bonner, 1952). It seems likely that the mechanisms of the stimulation of the oxidation of α-ketoglutarate and succinate are basically the same.

Expt. 2 of Table 4 shows that 10^{-4} M dinitrophenol is sufficient completely to inhibit the respiratory chain phosphorylation, even in the presence of 5 mg./ml. albumin.

Table 3. *Effect of α-ketoglutarate concentration on the activation of the oxidation of α-ketoglutarate by dinitrophenol*

Blowfly sarcosomes isolated with 0·32 M sucrose + 0·01 M ethylenediaminetetraacetate, pH 7·4, from 2-day-old blowflies.

$$\text{Ratio} = \frac{\text{value in presence of dinitrophenol}}{\text{value in absence of dinitrophenol}}.$$

	Dinitrophenol (M)	α-Ketoglutarate (m-moles/l.) 2·2	5·5	10·5
$-\Delta O$ (μatoms)	0	1·00	1·43	1·49
	10^{-4}	0·90	1·59	1·75
Ratio		*0·90*	*1·11*	*1·17*
$-\Delta\alpha Kg$ (μmoles)	0	0·41	0·69	—
	10^{-4}	0·50	1·01	—
Ratio		*1·22*	*1·46*	
Δ Esterified P (μatoms)	0	1·66	2·22	2·41
	10^{-4}	0·38	0·67	0·83
Ratio		*0·23*	*0·30*	*0·34*
P:O	0	1·66	1·55	1·62
	10^{-4}	0·42	0·42	0·47

Table 4. *Effect of dinitrophenol on oxidation of succinate by blowfly sarcosomes*

Expt. 1, same preparation of sarcosomes as in Fig. 3; Expt. 2, sarcosomes isolated in 0·32 M sucrose + 0·01 M ethylenediaminetetraacetate, pH 7·4 from 8-day-old blowflies. Reaction mixture same as for α-ketoglutarate, except that 0·01 M succinate replaced α-ketoglutarate, and fluoride and malonate were omitted.

Expt.	Dinitrophenol (M)	Albumin (mg./ml.)	$-\Delta O$ (μatoms)	Δ Esterified P (μatoms)	P:O
1	0	0	2·10	0·29	0·14
	10^{-4}	0	3·49	0·12	0·03
2	0	0	5·07	0·27	0·05
	5×10^{-6}	0	4·94	0·16	0·03
	10^{-5}	0	5·01	0·15	0·03
	2×10^{-5}	0	4·80	0·07	0·01
	5×10^{-5}	0	4·84	0·06	0·01
	10^{-4}	0	5·06	−0·05	−0·01
	0	5	5·74	1·82	0·32
	5×10^{-6}	5	6·15	1·59	0·26
	10^{-5}	5	6·17	1·45	0·23
	2×10^{-5}	5	6·10	1·22	0·20
	5×10^{-5}	5	6·16	0·63	0·10
	10^{-4}	5	5·94	0·09	0·01

DISCUSSION

Dinitrophenol-resistant oxidative phosphorylation

Judah (1951) and Hunter (1951) independently and at about the same time made the important observation that dinitrophenol did not completely uncouple phosphorylation when α-ketoglutarate was substrate. Since the phosphorylation (Hunter & Hixon, 1949) accompanying the Krebs & Cohen (1939) dismutation of α-ketoglutarate was little affected by concentrations of dinitrophenol which completely uncoupled phosphorylation associated with the oxidation of other substrates, Judah concluded that the substrate-linked phosphorylation was not affected by dinitrophenol. More recently this has been confirmed by Kaufman, Gilvarg, Cori & Ochoa (1953) and by Hift, Ouellet, Littlefield & Sanadi (1953), using partially purified soluble enzymes. These enzymes bring about the following reactions (CoA = coenzyme A; DPN = diphosphopyridine nucleotide; $DPN_{red.}$ = reduced diphosphopyridine nucleotide):

(*a*) $\alpha Kg + CoA + DPN \rightarrow$ succinyl-CoA $+ CO_2 + DPN_{red.}$

(*b*) succinyl-CoA $+ ADP + H_3PO_4 \rightleftharpoons$ succinate $+ ATP + CoA.$

(*a*) + (*b*) $\alpha Kg + DPN + ADP + H_3PO_4 \rightarrow$ succinate $+ DPN_{red.} + ATP.$

Both the rat-heart and the blowfly sarcosomes also have a dinitrophenol-resistant phosphorylation with α-ketoglutarate as substrate. In this respect, the blowfly sarcosomes differ from mung-bean mitochondria (Bonner & Millerd, 1953), which also give low P:O ratios. However, the P:αKg ratio in the presence of dinitrophenol is considerably below unity, the value expected if the substrate-linked phosphorylation were completely unaffected by dinitrophenol. Moreover, the P:αKg ratio in the presence of 10^{-4} M dinitrophenol varied considerably with different preparations. Hunter (1951) also obtained a low P:αKg ratio (about 0·5) with liver mitochondria in the presence of dinitrophenol.

Three possible explanations of the low ratios for the dinitrophenol-resistant phosphorylation will be considered. (1) The substrate-linked phosphorylation reaction is inhibited by dinitrophenol. (2) Dinitrophenol increases the ATPase activity, so that the phosphorylative activity is not completely measured. (3) In the presence of dinitrophenol, α-ketoglutarate is oxidized by a non-phosphorylating pathway as well as by that described in equations (*a*) and (*b*).

The first possibility is eliminated by the report of Hift *et al.* (1953) that the enzyme catalysing reaction (*b*) is not inhibited by up to 10^{-3} M dinitrophenol and also by the shape of the curves in Fig. 2. (Hunter's (1951) graph is very similar.) If dinitrophenol inhibited the substrate-linked phosphorylation reaction, the P:αKg ratio would decline progressively as the concentration of dinitrophenol increased. In Fig. 2 (it should be noted that the concentration is plotted on a logarithmic scale), it can be seen that further addition of dinitrophenol beyond 5×10^{-5} M in the case of blowfly sarcosomes or 10^{-4} M in the case of heart sarcosomes caused no further decline in the P:αKg ratio, until a concentration of 5×10^{-4} M was exceeded. Between 5×10^{-5} M and 5×10^{-4} M dinitrophenol, there was in fact a slight rise in the P:αKg ratio with the blowfly preparations, isolated in sucrose.

The second possibility can be excluded, so far as the heart sarcosomes are concerned, by the finding of Slater & Cleland (1953*a*) that 10^{-4} M dinitrophenol increased the ATPase activity by only 58%. The

amount of hexokinase used was considerably in excess of that required to react with virtually all the ATP, according to the analysis of Slater & Holton (1953), and a 58% increase in ATPase activity would cause a negligible loss. In any case, it is possible by adding albumin to obtain P:αKg ratios near unity in the presence of 10^{-4}M dinitrophenol.

This leaves the third possibility considered above. It is probable that the alternative pathway is provided by the substitution of reaction (*b*) by the hydrolysis of succinyl-CoA, catalysed by an enzyme isolated from heart by Gergely, Hele & Ramakrishnan (1952) and by Kaufman *et al.* (1953). This raises an important question. Does the succinyl-CoA deacylase operate in the untreated sarcosomes and so decrease the yield of phosphorylation available from respiration or does it become active only in the presence of dinitrophenol? The succinyl-CoA deacylase is probably present in the intact sarcosome, but it may not be accessible to the succinyl-CoA formed by the oxidative decarboxylation of α-ketoglutarate. While it is not possible to make a final choice between these two possibilities, the ability of dinitrophenol to increase the rate of other reactions catalysed by the sarcosomes (respiration, hydrolysis of ATP) suggests that it might increase the deacylase activity in the same way. This is given some support by the finding that although the P:αKg ratio in the presence of 10^{-4}M dinitrophenol is increased by albumin, the phosphorylation ratios in the absence of dinitrophenol are little affected by the addition of albumin (Lewis & Slater, 1954; see also Table 2 of the present paper).

On the basis of this explanation, it would be concluded from Fig. 2 that the deacylase would be completely activated (or become completely accessible to the succinyl-CoA) at about 5×10^{-5}M dinitrophenol. The slight increase of the P:αKg ratio often found with higher concentrations of dinitrophenol could be because the deacylase is inhibited by these concentrations.

Since 10^{-4}M dinitrophenol was sufficient completely to inhibit the respiratory chain phosphorylation associated with the oxidation of succinate, even in the presence of 5 mg. albumin/ml., it is clear that the higher P:αKg ratios obtained in the presence of 10^{-4}M dinitrophenol by adding 1·2 mg. albumin/ml. are due to a protection of the substrate-linked phosphorylation. This might be due to an effect on the structure of the sarcosome (cf. Watanabe & Williams, 1953) which results in the succinyl-CoA and the deacylase remaining inaccessible to one another. It has been shown (Lewis & Slater, 1954) that grinding the thoracic muscle in a mortar for only 1 min. caused a marked decline in the Q_{O_2} and in the P:O ratio of the isolated sarcosomes. Expt. 10, Table 1, shows that the P:αKg ratio of such a preparation in the presence of 10^{-4}M dinitrophenol was very low (0·32). This is probably associated with structural damage to the sarcosome.

Stimulation of phosphorylation by dinitrophenol

Fig. 3 illustrates an apparently anomalous situation in which the rate of esterification of inorganic phosphate by oxidative phosphorylation is increased by dinitrophenol, the typical inhibitor of oxidative phosphorylation. This result is a consequence of three factors: (1) the substrate-linked phosphorylation is little affected by dinitrophenol; (2) the dinitrophenol-sensitive respiratory chain phosphorylation contributes a relatively small proportion of the total phosphorylation in this preparation of sarcosomes from young blowflies; (3) dinitrophenol increases the rate of oxidation of α-ketoglutarate.

This experiment provides evidence in favour of the feasibility of the mechanism suggested by Lardy (1952) to explain the stimulation of metabolism by certain hormones. Lardy suggested that these hormones acted by partially uncoupling phosphorylation from respiration in such a way that the rate of the respiration was increased to a greater degree than the decrease of the P:O ratio. Thus, although the efficiency of the utilization of respiration for the synthesis of ATP would be decreased, the actual rate of synthesis of ATP would be increased.

Chin & Bueding (1954), working with a particulate fraction of *Ascaris lumbricoides*, have recently also found an increase of the rate of phosphorylation on the addition of dinitrophenol. The explanation in this case must be more complicated than that given above, since in Chin & Bueding's experiments the P:O ratio was considerably increased by dinitrophenol.

Mechanism of stimulation of respiration

The mechanism of respiratory chain phosphorylation previously proposed (Slater, 1953*b*) provides an explanation of the stimulation of respiration by dinitrophenol. In the following equations:

$$AH_2 + B + C \rightleftharpoons A \sim C + BH_2, \quad (1)$$

$$A \sim C + ADP + H_3PO_4 \rightleftharpoons A + C + ATP, \quad (2)$$

$$A \sim C + H_2O \rightarrow A + C, \quad (3)$$

AH_2 and B are adjacent members of the respiratory chain and C is an additional component or grouping required for their interaction. A and C must be regenerated from the compound $A \sim C$ before hydrogen-transfer can continue. In the intact mitochondria or sarcosome, this is brought about primarily by the transphosphorylating reaction (2), which leads to the esterification of inorganic phosphate. It is suggested that reaction (3) is relatively slow in the intact mitochondria, but can be greatly accelerated by dinitrophenol. In this way, $A \sim C$

would be hydrolysed and be unavailable for the synthesis of ATP by reaction (2).

When reaction (2) is fast compared with reaction (1), the addition of dinitrophenol by promoting reaction (3) will stop the esterification of phosphate but since it increases the rate of a process which is not rate-limiting, it will not increase the rate of hydrogen-transfer to any extent. This is presumably the situation in liver mitochondria or heart sarcosomes. It would be consistent with our experience with heart sarcosomes that the P:O ratio is largely unaffected by treatments which considerably affect the oxidative activity (Slater & Cleland, 1953*b*).

When, on the other hand, reaction (2) is slow compared with reaction (1), the rate of hydrogen-transfer will be increased by increasing the rate of breakdown of $A \sim C$, for example by the addition of dinitrophenol. This is probably the case with the sarcosomes of young blowflies isolated with media containing ethylenediaminetetraacetate.

Only preparations of blowfly sarcosomes which were isolated from young blowflies with media containing ethylenediaminetetraacetate showed the stimulation of respiration by dinitrophenol (Table 1), and different preparations within this group varied considerably. Although there was a considerable variation in the oxidative activity in the absence of dinitrophenol, only in one case (sarcosomes isolated in 0·16 M phosphate, pH 7·0, 2·5 % albumin) could the lack of stimulation be ascribed to the fact that the activity without adding dinitrophenol was already high. Table 1 shows no obvious correlation between the activation of the oxidation of α-ketoglutarate by dinitrophenol and the P:O ratio in the absence of dinitrophenol.

Two possible explanations of the failure of preparations made without ethylenediaminetetraacetate or from older blowflies to be activated suggest themselves:

(i) The potential activity of the α-ketoglutaric oxidase system, i.e. the activity of reaction (1) when $A \sim C$ is decomposed as fast as it is formed, is much lower in these preparations. This would imply that the potential activity of the α-ketoglutaric oxidase system in the blowfly sarcosomes declines rapidly during the first week of life. It would also mean that the potential activity was decreased when preparations were isolated in sucrose without ethylenediaminetetraacetate, in agreement with the experience with heart sarcosomes (Slater & Cleland, 1953*a*).

(ii) The activity of the enzyme catalysing reaction (3) is inadequate even when fully activated by dinitrophenol. This explanation is not necessarily inconsistent with the uncoupling of the respiratory chain phosphorylation by dinitrophenol, since it is possible that the $A \sim C$ block envisaged above occurs at a point in the respiratory chain where little phosphorylation occurs, because of the absence of the enzyme catalysing reaction (2). This is not unreasonable in view of the low P:O ratios of blowfly sarcosomes.

Fig. 2 shows that the concentration of dinitrophenol required to uncouple respiratory chain phosphorylation is less in the case of blowfly sarcosomes than in the case of the better phosphorylating heart sarcosomes. Moreover, the preparation of blowfly sarcosomes with the highest P:O ratio in the absence of dinitrophenol required the highest concentration of dinitrophenol for uncoupling. These relationships can be explained by the relative activities of the enzymes catalysing reaction (2); the greater the activity, the greater has reaction (3) to be stimulated so that all the $A \sim C$ is hydrolysed.

The concentrations of dinitrophenol required in the present study for stimulating respiration are much higher than those used by other workers using intact cells. This is understandable, since in the intact cell where [ATP]/[ADP] is high, the back reaction of equation (2) will be an additional factor limiting the breakdown of $A \sim C$. Under these circumstances, it can be shown by solving the kinetic equations appropriate to reactions (1), (2) and (3) that a moderate increase in the rate of reaction (3) will markedly increase the rate of oxidation. These equations also make it clear why higher concentrations of dinitrophenol are required to stimulate respiration than to uncouple phosphorylation.

In studies with the intact cell, concentrations above about 10^{-5} to 10^{-6} M dinitrophenol (e.g. Simon, 1953) inhibit respiration. No inhibitory effects were detectable with isolated sarcosomes below 10^{-3} M. This different behaviour is probably a reflexion of the different degrees of complexity of the two systems. In the intact cell, respiration may be governed by processes which require the supply of a small amount of ATP. In the simpler systems used in the present study, the medium contains all the components which are required for respiration and which are not in the sarcosome.

It has been found by a number of authors that respiration can proceed in the absence of phosphate acceptor, although usually at a lower rate (e.g. Lardy & Wellman, 1952; Slater & Holton, 1953). For respiration to occur, reaction (1) must proceed continuously in the one direction. This can be brought about only by introducing an irreversible reaction causing the decomposition of $A \sim C$ to A and C. There are only two possible irreversible reactions: (*a*) the irreversible hydrolysis of ATP by ATPase, or (*b*) the irreversible hydrolysis of $A \sim C$ (reaction (3)). There is no evidence which allows a choice between the two possibilities.

Lee & Eiler (1953*b*) have proposed a mechanism of respiratory chain phosphorylation which can be

rewritten in terms of the nomenclature used in this paper as follows:

$$AH_2 + B + P \rightleftharpoons A \sim P + BH_2, \quad (4)$$

$$A \sim P + ADP \rightleftharpoons A + ATP. \quad (5)$$

These authors believe that inorganic phosphate is returned to the system by reversal of the reactions leading to its incorporation into ATP. Since this mechanism does not include the essential irreversible reaction mentioned above it is a closed circuit, describing the overall equilibrium

$$AH_2 + B + ADP + P \rightleftharpoons A + BH_2 + ATP. \quad (6)$$

The forward reaction is oxidative phosphorylation, the reverse is named reductive dephosphorylation. Even if the overall reaction describing the oxidation of substrate (SH_2) through A and B by oxygen is made irreversible by introducing reactions (7) and (8),

$$SH_2 + A \rightarrow S + AH_2, \quad (7)$$

$$BH_2 + \tfrac{1}{2}O_2 \rightarrow B + H_2O, \quad (8)$$

the overall reaction (9) (which is the sum of reactions (6), (7) and (8))

$$SH_2 + \tfrac{1}{2}O_2 + ADP + P \rightarrow S + H_2O + ATP \quad (9)$$

will stop as soon as all the ADP is phosphorylated. Thus Lee & Eiler's experiments, in which a steady respiration was obtained in the presence of added ATP, are not explained by their mechanism.

If the irreversible reaction permitting respiration is reaction (3), we can rewrite equations (1), (2) and (3) as follows

$$AH_2 + B + C \overset{(1)}{\rightleftharpoons} BH_2 + A \sim C \overset{(3)\ H_2O}{\longrightarrow} A + C$$

$$\begin{array}{l} \text{ADP} \\ + \\ H_3PO_4 \end{array} \Big\downarrow \quad \Big\uparrow (2)$$

$$A + C + ATP$$

This shows that reaction (2) will remain at the position of equilibrium throughout the reaction and consequently [ATP]/[ADP] will remain constant. This will be the case even after the addition of dinitrophenol, which will increase the rate of reaction (3) and consequently decrease the steady-state concentration of $A \sim C$. As a result, the rate of incorporation of ^{32}P into ATP will be decreased without altering the concentration of ATP. This is what Lee & Eiler (1953*a*) found experimentally and explained (Lee & Eiler, 1953*b*) in essentially the same way.

But this is not the only possible explanation of Lee & Eiler's findings. If, as is more generally believed, the irreversible reaction permitting respiration is the hydrolysis of ATP, it would certainly be expected that dinitrophenol in an amount sufficient to depress the phosphorylation would decrease the concentration of ATP. But if, as is the case in Lee & Eiler's experiments, the ratio [ATP]/[ADP] is, in the absence of dinitrophenol, so high that ADP cannot be detected, an inhibition of the phosphorylation by 40% (the value obtained by Lee & Eiler (1953*a*)) might result in a considerable decrease in the ratio [ATP]/[ADP], without decreasing [ATP] sufficiently to be detected experimentally.

SUMMARY

1. The rate of oxidation of α-ketoglutarate and succinate by some preparations of blowfly sarcosomes was markedly increased by 2:4-dinitrophenol, even in the presence of excess hexokinase and glucose.

2. Only preparations isolated from young blowflies in media containing ethylenediaminetetraacetate showed this effect. The respiration of sarcosomes isolated from older blowflies, or from younger flies in sucrose medium, was little affected by dinitrophenol. The respiration of heart sarcosomes was also unaffected.

3. In agreement with earlier work of others, it was found that there was a dinitrophenol-resistant phosphorylation step in the oxidation of α-ketoglutarate. This is undoubtedly the substrate-linked phosphorylation, involving succinyl-coenzyme A.

4. The phosphorus:α-ketoglutarate ratio in the presence of sufficient dinitrophenol completely to inhibit respiratory chain phosphorylation was substantially below the theoretical value of 1, but could be raised to near 1 by the addition of albumin, which did not prevent the inhibition of respiratory chain phosphorylation by dinitrophenol.

5. With some preparations, the increase of respiration brought about by dinitrophenol more than counterbalanced the decrease in the phosphorylation ratios, with the result that the rate of phosphorylation was actually increased by dinitrophenol.

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Synthesis of Cellulose by *Acetobacter xylinum*

2. PREPARATION OF FREEZE-DRIED CELLS CAPABLE OF POLYMERIZING GLUCOSE TO CELLULOSE*

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Non-proliferating cells of *Acetobacter xylinum* acting on glucose in the presence of oxygen are known to synthesize cellulose (Hestrin, Aschner & Mager, 1947; Hestrin, 1953). Hitherto, however, the factors determining rate and yield of the production of cellulose in this system have not been described quantitatively. In an earlier communication, a micromethod for the specific determination of cellulose in a biological mixture was presented (Schramm & Hestrin, 1954*a*). The present paper describes the procedure for the separation of *A. xylinum* bacteria from their cellulose pellicle and the preparation of an essentially cellulose-free suspension of non-proliferating freeze-dried cells which polymerize glucose to high-polymer cellulose. Optimum conditions for the synthesis are described and the specific synthetic activity of the cells is evaluated. Some observations which relate to the mechanics of the growth of cellulose gel bodies are also presented.

The chemical mechanism of the synthesis of cellulose by *A. xylinum* will be the subject of a later communication.

* Experiments described in this communication were carried out by M.S. in the course of work leading to the Ph.D. degree.

METHODS AND MATERIALS

Culture technique

The following medium was used (%, w/v): glucose, 2·0; peptone (Difco Bactopeptone), 0·5; yeast extract (Marmite), 0·5; disodium phosphate (anhydrous), 0·27; citric acid (monohydrate), 0·115; pH adjusted to 6·0 with dil. HCl or NaOH. The solution was cleared by filtration and sterilized by autoclaving. A shallow-layer quiescent-culture technique which favours rapid appearance of a surface pellicle and growth of the vigorously cellulose-synthesizing *A. xylinum* wild type (Schramm & Hestrin, 1954*b*) was used. The medium was inoculated 1:10 with fluid from the liquid phase of a 3-day starter culture, distributed aseptically into large Petri dishes as a shallow fluid layer and incubated at 30° at rest. The pellicles were harvested after 48 hr.

Preparation of freeze-dried bacteria

The separation of *A. xylinum* bacteria from their matrix of cellulose was based on the observation that cheesecloth retains the cellulose fibrils but allows the bacteria to pass (Hestrin *et al.* 1947). Sodium phosphate–citrate mixture (pH 6·0; final phosphate concn., 0·01 M) was used as buffer. At all stages of washing, the temperature of the milieu was kept below 6° with ice. The use of this low temperature both conserved activity and protected the bacteria from agglutination in bodies of cellulose gel which might otherwise have formed during the early stages of the washing process from the traces of glucose in the cell milieu.

The pellicles were cooled, drained and then pulped rapidly in a Waring Blendor (1 min. at high speed) under 5 vol. of buffer. The foam was skimmed off and the suspension of bacteria and fibrils was passed through 4 layers of cheesecloth. Residues on the cloth and the separated foam were stirred into fresh buffer and the procedure was repeated. The filtrates were centrifuged giving a pink paste. With the aid of some glass beads the paste was dispersed and washed in buffer twice by centrifuging. Finally the suspension was passed first through 6 layers of cheesecloth and then through glass wool. The filtrate was centrifuged and the bacteria were taken up in 5 vol. of buffer and freeze-dried using a low-temperature trap as moisture receiver. The dry light-brown product was stored at $-20°$ in evacuated ampoules. It readily dispersed in water or in buffer solutions to yield a silky, fairly stable suspension.

The recovery of dried cells was of the order of 150 mg./l. of medium. This corresponded to about 62% of the dry wt. of the water-rinsed pellicle. There were approximately 10^9 cells/mg. of the powder (cells enumerated in a blood-count chamber). The nitrogen (Kjeldahl) content of the powder was 12% and the apparent cellulose content (method of Schramm & Hestrin, 1954*a*) $0{\cdot}5 \pm 0{\cdot}1$%.

Storage in the air-free ampoules at $-20°$ for 2 months did not result in marked loss of the activity. When the powder was stored under air at room temperature, the activity declined markedly within two days.

Measurement of synthetic activity

The standard reaction mixture was made up to a total volume of 20 ml. in a 125 ml. stoppered Erlenmeyer flask to contain: freeze-dried cells, 10 mg.; glucose, 0·2M; sodium citrate, 0·02M, pH 6·4; gas phase, O_2. The reaction flask was shaken reciprocally at a rate of 100 cyc./min. (90° arc) in a water bath at $30 \pm 0{\cdot}1°$. The reaction was terminated (after 1–3 hr.) by adding enough ether to cover the surface.

Analytical methods

Cellulose was determined as previously described (Schramm & Hestrin, 1954*a*). The cellulose content at zero time was deducted from the total yield to obtain the amount formed.

Oxygen uptake was measured manometrically in a Warburg flask with an alkali wick in the centre well.

The radioactivity of ^{14}C was measured with a thin-window Geiger-Müller tube. The glucose solution for the measurement was taken up in a disk of filter paper and dried in the planchet. The cellulose sample for measurement was purified as described by Schramm & Hestrin (1954*a*) and spread out in a film on the bottom of the planchet so that it had roughly the same dimensions and form as the filter-paper disk. The counts were corrected for self-absorption. After the measurement of radioactivity, the amount of the cellulose in the sample was determined chemically. When a non-radioactive sample of cellulose was purified after prior immersion in [^{14}C]glucose solution, the radioactivity of the purified specimen was negligible.

Mechanical agitation

Reciprocal motion was produced on a wrist shaker (90° arc; 100 cyc./min.) and swirling motion on a Ross-Kershaw apparatus (A. H. Thomas and Co., Philadelphia, U.S.A.).

Measurement of infrared absorption

A foil of the synthesized cellulose was used. This was prepared by incubating the standard reaction mixture for 20 hr. without agitation. The foil was rinsed in water, purified by storage in 1% NaOH, and rinsed again in water. The infrared absorption spectrum was kindly worked out by Drs J. Rowen and R. Nusbaum, Physiological Chemistry Department, Medical School, University of California, U.S.A., according to the procedure of Forziati & Rowen (1951) with the aid of a fine-grating instrument of high resolving power. The reference for comparison was a somewhat thicker foil which had been obtained from the surface of a 48 hr. culture of *A. xylinum* (cf. also, Shirk & Greathouse, 1952).

RESULTS

Synthesis of cellulose from [^{14}C]*glucose*

Synthesis of the cellulose from added glucose was demonstrated unambiguously by using [^{14}C]glucose as a substrate. The radioactivity of the cellulose was between 78 and 88% of the value required on the assumption that the carbon of the cellulose is derived solely from the added glucose. An experiment in triplicate is presented in Table 1. While the possibility cannot be excluded that carbon from an unknown endogenous source participated in cellulose synthesis, it is clear that the major carbon source was the exogenous glucose. Synthesis of radioactive cellulose from [^{14}C]glucose has been described previously in the cotton boll (Greathouse, 1953) and in growing cultures of *A. xylinum* (Minor, Greathouse, Shirk, Schwartz & Harris, 1954) and *A. acetigenum* (Bourne & Weigl, 1954).

Kinetic properties of the polymerizing system

Glucose and oxygen supplied together were necessary for synthesis of cellulose by the freeze-dried cells. When the latter had been prepared with rigid adherence to the procedure described, the apparent cellulose content of the standard reaction mixture at zero time was a small fraction ($\leqslant 10$%) of the total found at the end of 2 hr. When the cells were shaken, either under oxygen in the absence of glucose or under nitrogen (pure, or admixed with

Table 1. *Synthesis of cellulose from* [^{14}C]*glucose*

[^{14}C]Labelled glucose (36·8 μmoles; total counts, 5600/min.) and dried cells (10 mg.) in total vol. 3·6 ml. Conditions otherwise standard. Reaction time, 2 hr.

Expt. no.	Cellulose formed (μmoles glucose)	Radioactivity of cellulose: Total (counts/min.)	Radioactivity of cellulose: % of initial activity*
1	3·72	498	88
2	3·63	483	87
3	3·49	456	86

* Specific activity of polymerized glucose as % of that of added glucose (counts/min./μmole).

carbon dioxide) in the presence of glucose, the low initial apparent cellulose content of the cells remained constant. In the absence of glucose the O_2 uptake rate, too, was negligible, being less than 0·01 μmole/mg./hr. In the presence of glucose the O_2 uptake rate was about 4 μmoles/mg./hr.

The course of the synthetic reaction under the standard test conditions is illustrated by Fig. 1. The rate was maximal from the outset. There was a relatively rapid early decline. Thereafter, the rate either remained practically constant or declined slowly over a period of several hours. The pH was held during this time within 0·1 pH unit of the initial value, as the capacity of the citrate buffer was sufficient to neutralize the acids (chiefly gluconic) formed oxidatively by the cells from glucose. Variation of glucose concentration within a wide range (3–200 mM) was without effect on the initial rate of the synthesis. Variation of shaking rate (80–120 cyc./min.) did not alter the synthesis course and rate.

The plot of the amount of cellulose formed against the amount of cells was a straight line which passed through the origin (Fig. 1). Replicate assays agreed within ± 3·5 %. In the tested range of cell concentration (⩽ 1 mg./ml.) and incubation time (1–3 hr.), specific synthetic activity (μmoles glucose polymerized/mg. cells (dry wt.)/hr.) could thus be calculated by linear extrapolation. The cellulose-synthesizing power of the freeze-dried cells was 50–100 % of that of the freshly harvested cells. Under standard conditions the specific synthetic activity of the freeze-dried preparation fell in the range of 0·1–0·25 μmole/mg./hr., the value depending on the batch.

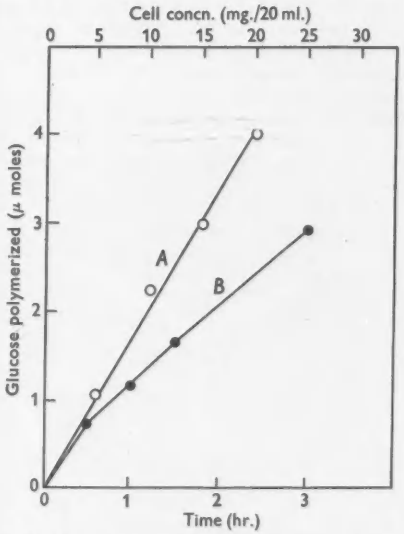

Fig. 1. Influence of reaction time and cell concentration on cellulose production. Curve *A* shows the glucose polymerized as a function of cell concn.; curve *B* shows the glucose polymerized as a function of time. The reaction time was 1·5 hr. for curve *A*. The cell concn. was 0·5 mg./ml. for curve *B*. Conditions otherwise were standard. Experiments involved two different batches of cells.

The complete course of the synthesis was recorded for the case of limiting glucose concentration (Fig. 2). In such a reaction mixture, when the rate of synthesis in this reaction mixture had become negligible at the end of an incubation time of 4 hr., chromatographic analysis showed no glucose. Since the cellulose formed was not decreased when incubation was continued after no glucose remained, the overall process of the synthesis can be regarded as essentially irreversible. The terminal yield of the cellulose was 26 % of the glucose initially added. This yield is several-fold higher than any recorded hitherto for cellulose synthesis either by *A. xylinum* or any related species (cf. Tarr & Hibbert, 1931; Kaushal & Walker, 1951).

The effect of O_2 tension is illustrated in Fig. 3. The synthesis velocity with glucose in excess was about half-maximal at O_2 tension 20 % and gradually approached maximum at tension 100 %. The sensitive dependence of the synthesis velocity on O_2 tension explains in part why the production of cellulose in an undisturbed culture of *A. xylinum* is restricted to the liquid–air interphase (Schramm & Hestrin, 1954*b*).

The effect of pH on the rates of the polymerization and oxidation are compared in Table 2. Both the polymerization and O_2 uptake were maximal in the pH range 5–7. The rate of the polymerization is retarded relatively to O_2 uptake in the pH region below 5.

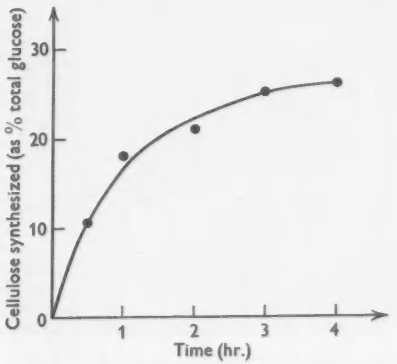

Fig. 2. Production of cellulose in reaction mixture containing limiting concentration of glucose. The digest contained 10 mg. dried cells and 10 μmoles glucose in total vol. 2·0 ml. Conditions otherwise standard.

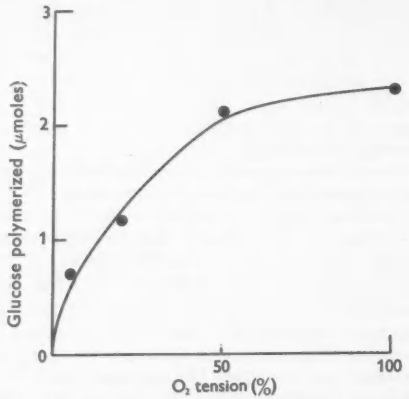

Fig. 3. Influence of O_2 tension on synthesis velocity. O_2 tension was varied by diluting gas phase with nitrogen. The digest contained 15 mg. dried cells and McIlvaine's phosphate–citrate buffer (pH 6·0; diluted 12-fold). Conditions otherwise standard.

Table 2. *Rates of cellulose synthesis and oxygen uptake of freeze-dried cells of* Acetobacter xylinum *as a function of* pH

Conditions were standard except for use of sodium phosphate–citrate mixture as buffer (final concn. as to phosphate, 0·01 M). The O_2 uptake was determined for the period 0·5–1·0 hr.

pH		Activity (μmoles/mg./hr.)		
Initial	Final	Glucose polymerized (*a*)	O_2 uptake (*b*)	*b*/*a*
2·9	2·9	0·04	3·2	80
4·0	3·9	0·12	4·5	38
5·2	4·7	0·21	4·2	20
6·4	6·0	0·20	4·8	24
7·3	6·9	0·22	4·2	19
8·5	7·3	0·12	3·2	27

Table 3. *Qualitative properties of cellulose formed from glucose by freeze-dried cells of* Acetobacter xylinum

Colour reactions

(*a*) Test for cellulose with iodine–zinc chloride reagent: positive (blue-violet stain).

(*b*) Reduction of copper reagents: negative prior to hydrolysis; positive after hydrolysis.

(*c*) Test for glucose with phosphoric acid–pyrocatechol reagent (Hestrin & Mager, 1947): positive.

(*d*) Test for non-glucose sugars with phosphoric acid (Hestrin & Mager, 1947): negative.

Solubility tests

(*a*) Liquids with dispersing action: acetic anhydride (in presence of catalyst); conc. mineral acids (H_2SO_4; HCl; H_3PO_4); Schweizer's reagent (slow action).

(*b*) Liquids without dispersing action: common organic solvents (ethanol, ether, acetone); weak acids and dil. strong mineral acids (5 N-H_2SO_4, glacial acetic acid); aqueous alkali (N and 5 N-NaOH).

Identification of polymerization product

The general appearance and behaviour of the polymerization product closely recall the gel produced from glucose by *A. xylinum* in culture. The product has been identified unambiguously on the basis of the infrared absorption spectrum which was kindly prepared by Drs Rowen and Nusbaum (Fig. 4). The material on this basis can be regarded as high-polymer, native, crystalline (type I) cellulose. As has been pointed out by Dr Rowen in a personal communication, the identity of the absorption peaks found with synthetic and natural cellulose of *A. xylinum* in the spectral region 2750–3750 cm.$^{-1}$ suggests that even the fine-structure hydrogen bonding of these specimens is identical. A detailed report will be presented by Drs Rowen and Nusbaum elsewhere.

Supplementary observations confirming the identification of the product are assembled in Table 3. An X-ray diffraction diagram of the

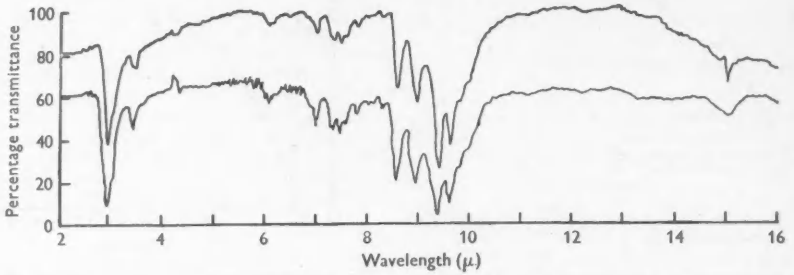

Fig. 4. Infrared absorption spectrum of cellulose foil produced from glucose by freeze-dried *Acetobacter xylinum*. For details of procedure see under Methods and Materials. The upper curve is for cellulose foil synthesized by freeze-dried cells; the lower curve is for cellulose foil from culture of *A. xylinum*.

synthetic cellulose was obtained by Drs Alexander and Fraenkel, Department of Physics, Hebrew University, Jerusalem, Israel, and found to be identical in all important respects with a diagram afforded under the same conditions by cotton.

Polysaccharide products other than cellulose (the alkali-insoluble fraction) have not been detected. A water-soluble extract of a reaction mixture after 2 hr. incubation was devoid of any material precipitable by ethanol (80 %, v/v). On hydrolysis in 2N sulphuric acid for 1 hr. in a boiling-water bath, the fraction insoluble in water and soluble in hot 4 % sodium hydroxide gave approximately the same yield of reducing sugar as a corresponding fraction prepared from cells that had been incubated without glucose. The water-insoluble alkali-soluble polysaccharide content of *A. xylinum*, as measured by the anthrone method of Fales (1951), was 17 μg./mg. On the incubation of *A. xylinum* with glucose during 2 hr. under the conditions of the standard reaction mixture, this value was not changed significantly.

Gross morphology of gel

Gross alterations in the appearance of the reaction mixture began immediately after the addition of glucose. These did not occur in the absence of either glucose or oxygen. The alterations followed a characteristic sequence (Fig. 5): silky suspension → loose fluff → floccules → large bodies. The bulk of the cells present initially free in suspension was incorporated in the formed gel. The final form assumed by the latter was a function of the intensity and type of agitation applied during reaction. The product of an undisturbed reaction mixture was a single, thin floating foil. That of a mixture agitated by reciprocal shaking was a single body of irregular form (Fig. 6). Several spherical and stellate bodies were afforded terminally by a swirled reaction mixture (Fig. 7).

Fig. 5. Appearance of cell suspension at successive times of reaction. Conditions standard. Mixture was photographed in a Petri dish over a dark background. Sectors *a*, *b*, *c* are from photographs taken 0, 5 and 30 min. respectively following initiation of reaction by glucose. ×1.

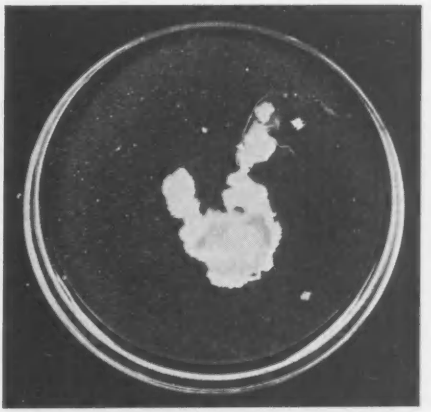

Fig. 6. Single gel body formed in reciprocally shaken reaction mixture. Conditions standard. Suspension was photographed in a Petri dish against a dark background at reaction time 3 hr. Note piece of fragile membrane attached to the gel body on the right-hand top. This was formed after the shaking had been terminated.

Fig. 7. Stellate gel bodies formed in swirled reaction mixture. Conditions standard except that mixture was agitated by swirling. Suspension was photographed in a Petri dish against a dark background at reaction time 4 hr. Note the fine radial arms. ×0·8.

The synthesis continued without conspicuous fall of rate for long after the gel had become macroscopically visible and after it had tightly incorporated the bulk of the initially free bacteria. Thus the bacteria continue to synthesize cellulose even after their incorporation in the gel. Hence the gel framework must be penetrated readily by glucose and oxygen. As additional chains are intercalated, the gel may be expected to acquire increased tensile strength. This is observed.

The relative shares in synthesis of cells inside and outside the gel will be discussed elsewhere.

Microscopic morphology of gel

The microstructure of a cellulose gel produced by growing cells of *A. xylinum* has been described by a number of investigators (for electron microscopy see Franz & Schiebold, 1943; Frey-Wyssling & Mühlethaler, 1946; Mühlethaler, 1948, 1949; Kaushal, Walker & Drummond, 1951; Ranby, 1952*a*; for dark-field microscopy see Aschner & Hestrin, 1946).

Electron micrograms of specimens of gel formed by freeze-dried *A. xylinum* acting on glucose are reproduced in Fig. 8, with the kind permission of Drs Hirschfeld and Volcani of the Weizmann Institute of Science, Rehovot, Israel. The bacteria are seen enveloped by their polymerization product. The matrix appears in most parts to be amorphous. Fibrous strands are sometimes discernible within the sheet. Often fibres are seen crossing a torn area.

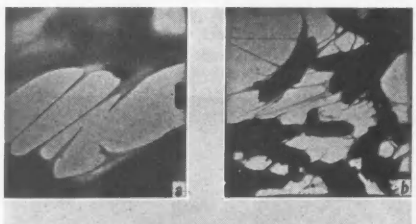

Fig. 8. Electron micrographs of gel formed from glucose by freeze-dried *Acetobacter xylinum*. Mixture agitated with a stream of oxygen at 20°. Glucose, 0·003 M. Conditions otherwise standard. After 15 min. a small gel body was picked up on to a stainless-steel grid (200 mesh), dried, rinsed in water, and examined under an RCA electron microscope, model EMU-2 A; *a* and *b* magnification about ×3600; *c* magnification about ×15 600. Dark shadows represent dried bacteria.

The predominantly amorphous structure of dried material is consistent with the suggestion that the framework of the native gel is a loose three-dimensional reticulum whose strands individually are mostly below the limit of resolution of the microscope. We have no evidence from the present study that the initially formed bacterial cellulose product consists largely of units 50–300 Å such as are present abundantly in well-crystallized cellulose specimens (Ranby, 1952*b*; Preston, 1952).

Influence of freeze-drying on viability of cells

Graded amounts of freshly harvested and freeze-dried cells were inoculated into 5 ml. of sterile nutrient medium. Presence of viable cells was revealed by the production of a surface pellicle within 2 weeks at 30° in undisturbed medium. Whereas the minimum amount of the freshly harvested bacteria giving a positive growth test was 10^{-9} mg., that of the freeze-dried bacteria was 10^{-7} mg. This suggests that the drying had resulted in the loss of viability of $>90\%$ of the cells. Loss of specific synthetic activity on drying was $<25\%$ in the same experiment. Nitrogenous nutrients (peptone, yeast extract) did not affect either the specific synthetic activity or the course of the reaction in the standard conditions during 3 hr. Whereas synthesis occurs at 37° as well as at 30°, cell growth in nutrient medium was rapid at 30° but was suppressed at 37°.

These findings suggest that neither an adaptive nor a proliferative increase of the enzymes concerned in cellulose production complicates the course of the synthesis of cellulose under the conditions of the standard reaction mixture.

DISCUSSION

In structure and composition the cellulose gel formed by *Acetobacter xylinum* formally resembles the green-plant primary wall (cf. Frey-Wyssling, 1952; Preston, 1952). As in the primary wall, the main mass is water, the framework is a spacious mesh of cellulose; centres of synthesis are discrete within the network, and the addition of cellulose can occur both within the gel and on the outer surface. The process of cellulose production by dried *A. xylinum* thus affords a system in which one or more of the terminal steps of the process of wall production occurs under controlled environmental conditions. Moreover, the chemistry as well as the mechanics of cellulose production by a green plant and by *A. xylinum* have points of similarity. Thus it has been shown that isolated tissues of a green plant require exogenous carbohydrate (glucose) and oxygen for net production of cellulose (Brown, Reith & Robinson, 1952).

For convenience as a research tool, *A. xylinum* presents distinctive merits of which the more important are the following:

(1) The rate of production of cellulose by the bacteria is of the same order of magnitude as the rates of production of reserve polysaccharides by slices of animal tissues, the activities being calculated to the basis of tissue dry weight.

(2) The synthetic activity withstands freeze-drying and subsequent prolonged storage.

(3) The cells are cellulose-free and readily dispersible.

(4) The endogenous rates of polymerization of glucose and uptake of oxygen are negligibly low.

(5) The cellulose formed by the bacteria is a well-known standard type, the crystalline high-polymer group which has been represented classically by the α-cellulose of cotton.

(6) The celulose is unencumbered by lignin, hemicellulose, pectin or other incrusting substances.

(7) The synthesis occurs from a defined substrate in a system which is practically free from net synthesis of any other polysaccharide.

(8) The reaction occurs under controlled conditions in a mixture of definite composition and follows a simple kinetic course uncomplicated by cell proliferation or adaptive enzyme synthesis.

(9) The polymer product is accumulated exclusively in the extracellular phase and there can readily be visualized.

At the electrolyte concentration of cellular fluids, hydrophobic micelles of high-polymer cellulose precipitate (Ranby, 1952*b*) and thus might be expected to accumulate near the locus of production. However, the cells of *A. xylinum*, as extracted mechanically from their pellicle, are practically cellulose-free (apparent cellulose $\sim 0{\cdot}5$ % of the total dry weight). Since cellulose chains are not accumulated within the cells, the possibility that the chains are formed extracellularly must be considered.

The apparent non-accumulation of cellulose in the cell interior suggests strongly that cellulose itself is not formed within the cells. The same finding tends also to discourage the notion that a spontaneously polymerizable precursor of cellulose, if indeed such a precursor exists in this system, is one formed inside the cells, as it would be necessary in that case to make the additional assumption that the cells possess a specific mechanism for prevention of spontaneous polymerization of the precursor in the intracellular phase. There has been a suggestion that the production of cellulose in *A. xylinum* depends on an extracellular enzyme (Mühlethaler, 1949). It has been shown above, however, that the cells form cellulose immediately on contact with glucose and oxygen even after thorough washing. If an extracellular enzyme had been necessary for the synthesis, the washed cells would have been either inactive or active only after a lag period.

Thus it seems probable that the terminal catalyst of the enzyme complement concerned in cellulose production is situated on the outer bacterial surface. On this view the cell membrane of *A. xylinum* could be regarded as resembling an inverted cytoplasmic membrane of a green-plant cell. The product of the surface reaction would have to be either a finished cellulose chain or a spontaneously polymerizable precursor of such a chain. In either case the ultimate orientation of the polymer chains as fibrils would be a non-enzymic process occurring in the extracellular phase.

On the estimate that there are 10^9 bacteria/mg. in the preparation of *A. xylinum*, the observed highest level of the specific synthetic activity of the bacteria ($0{\cdot}25$ μmole glucose polymerized/mg./hr.) corresponds to an hourly polymerization rate of the order of $1{\cdot}5 \times 10^9$ glucose residues per cell. The surface area of a bacterium is about $1\ \mu^2$. A cellulose chain $1\ \mu$. long consists of about 2×10^3 glucose residues. On this basis there should be room on the surface of a bacterium for about 4×10^6 glucose residues. This would lead to the estimate that the rate of production of cellulose on the bacterial surface under standard reaction conditions is of the order of 10^2 monomer monolayers/hr.

SUMMARY

1. A procedure for the preparation of *Acetobacter xylinum* in the form of a cellulose-free suspension of washed freeze-dried cells is described in detail.

2. Non-viable freeze-dried cells in optimum reaction conditions polymerized glucose to cellulose at a rate of $0{\cdot}25$ μmole/mg./hr., corresponding to a production of cellulose on the bacterial surface at a rate in the order of 10^2 monomer monolayers/hr.

3. Synthesis was attended by a rapid decline in the turbidity of the cell suspension and formation of a macroscopic cellulose gel body. The gross shape of the latter was a function of the type and intensity of the agitation during synthesis.

4. The polymerization product has been characterized on the basis of solubility, qualitative chemical properties, infrared absorption, X-ray diffraction, and electron micrography, and identified as high-polymer, native, crystalline (type 1) cellulose.

5. At least 78 % of the carbon of the polymer product was derived from exogenously added glucose. Synthesis was negligible in absence of glucose or oxygen. Synthesis was immediate in onset, progressive in course, variable linearly in rate with cell concentration and essentially irreversible. The optimum pH was in the range 5–7. The rate was about half-maximum at oxygen tension

20 % and approached maximum at tension 100 %. At low initial glucose concentration, the terminal yield of cellulose was 26 % of the total glucose.

6. Whereas the enzyme system for synthesis largely withstood freeze-drying, the latter incapacitated > 90 % of the cells for proliferation.

7. Kinetics of synthesis, properties of the cellulose, and the negligible cellulose content of the cells suggest that a constituent of the outer surface of the bacteria is a terminal catalyst of the production of cellulose.

8. Similarities between cells of *A. xylinum* and green plants and the use of *A. xylinum* as a model for the study of cell-wall production are evaluated.

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